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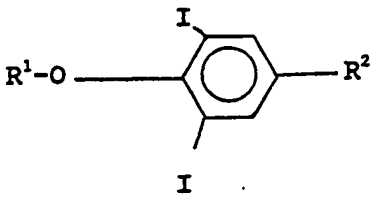
(54) Title: SELECTION METHOD FOR PHARMACOLOGICALLY ACTIVE COMPOUNDS



(I)

(57) Abstract

The present invention provides a predictive method for selecting organic compounds having useful pharmaceutical activity in a mammal. The method uses volume areas of similar molecules. When specific substituents of the test compound fit predetermined volumes, the compound is subject to a binding assay. Only when the binding assay is positive, is the experimental compound recommended for further pharmaceutical evaluation. Example compounds are derivatives of 4-substituted 2,6-diiodophenols of structure (1), where R^1 and R^2 are defined as aliphatic or substituted aliphatic moieties or aromatic or substituted aromatic moieties. Useful structures are described. The compounds have useful pharmaceutical properties to treat conditions in a human being, including hyperthyroidism, angina pectoris, arrythmia, and the like.

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SELECTION METHOD FOR PHARMACOLOGICALLY ACTIVE COMPOUNDS

BACKGROUND OF THE INVENTION

This is a continuation-in-part of the U.S. Patent application Serial No. 294,372, Filed on January 6, 1989 and also U.S. Patent Application Serial No. 295,041, filed January 6, 1989, both of which are incorporated herein by reference.

Field of Invention

The present invention relates to a method for selecting organic compounds having useful pharmacological activity in a mammal. More specifically, the present invention provides a method for selection of pharmacologically active compounds which are 4-substituted and hydroxyl substituted derivatives of 2,6-diiodophenol according to specific spatial atom arrangements. These compounds which may themselves be novel, are designed to produce pharmaceutical activity. In alternative, the evaluation of known compounds according to a novel method of this invention may discover new pharmacological activity and utility.

Related Disclosures

Many pharmacologically active drugs act on the cellular receptor level by either mimicking the action of a natural signal molecule (agonist) or by blockingXthe action of the natural signal molecule (antagonist).

Natural signalling molecules are endogenous compounds which chemically effect receptors located either on the

exterior of the cell membrane or the interior subcellular structure. For example, under the normal physiological conditions there is a certain level of a neurotransmitter or signal molecule released and/or present in the vicinity of the receptors. When, for any reason, such level is disturbed, that is when there is either the excess, deficiency or lack of neurotransmitter or signalling molecule, pathological conditions such as depression, schizophrenia, Parkinson's disease, Huntington's chorea, Grave's or Cushing's disease and many other debilitating conditions may develop.

Consequently, most cell receptors have a developed pharmacology of agents that act as agonists or antagonists. For example, suitable antagonists are known which can block the actions of transmitters dopamine, adrenalin, noradrenalin and acetylcholine or dopaminergic, alpha and beta adrenergic, and cholinergic agonists. Antagonists have been described in Pharmacological Basis of Therapeutics, 7th Ed., MacMillan, N.Y. (1985) acting on the endocrine molecules interacting with mineralocorticoid, glucocorticoid, estrogen and progesterone receptors. It is surprising, however, that despite extensive pharmacological research and development of many new methodologies and laboratory techniques, certain receptors, and/or their action still remain elusive and no antagonists have been yet discovered to inhibit or modulate their activity. Thyroid hormone receptors are one of them and despite numerous structure activity studies conducted over the past 20 years,

no antagonist for thyroid hormone receptor action has been identified.

Thus, it would be advantageous to have available method which would, based on certain chemical spatial arrangements provide, and allow the design of new chemical materials which would match, complement, partially block, completely inhibit, modify, accentuate or otherwise alter or effect the function of known receptors.

Numerous structure-activity studies of various endogenous chemicals and pharmaceutical drugs have suggested the necessity of bulky iodine or propyl substituents on the outer ring 3' position and the presence of a carboxylic acid group for effective receptor thyroid hormone binding and/or agonist function. Hormonal Proteins and Peptides, VI, 107-204 (1978), Academic Press, N.Y.; Endocrine Rev., 1:140-166 (1980).

Thyroid hormones thyronine (T4) and triiodothyronine (T3) affect the growth, development and metabolism of virtually all tissues of higher organisms. Since these hormones are endogenous, they act as agonists on the thyroid gland cell receptors known as iodothyronine receptors.

Recent studies summarized in Proc. Nat. Acad. Sci., 70:3488 (1973) have demonstrated that T4 is converted to T3 by deiodination in vivo which suggest that T4 functions as a prohormone, and all T4 biological activity, in fact, results from its conversion to T3 in vivo. High-affinity limited capacity thyroid hormone receptors have been

identified in the nuclei of most tissues and the specific association between thyroid hormone and thyronine binding globulin and prealbumin, two proteins responsible for transport of the thyroid hormones to tissue sites, have also been described in Biochemistry, 21-163 (1982).

T3 and T4 induce a maximal 4-fold increase in the rates of growth and glucose utilization of GH_1 cells, a pituitary tumor cell line in cell culture. Binding studies of T3 and T4 to cellular fraction showed high-affinity, low capacity binding sites for the hormones in nuclear but not mitochondrial or cytosol fractions of the cell.

In view of the above studies, any compound which would act on iodothyronine receptors should meet the structural requirements, i.e., bulky iodine or propyl substituent on the outer 3-ring position and the presence of a carboxylic acid group together with the ability to bind to a fraction of thyroid hormone receptors.

It has, however, been recently reported that certain chemical compounds which possess neither of the required moieties nor the obvious structural similarities with T3, T4, when their two-dimensional chemical structures are compared with these compounds, do show hyper- and hypothyroid-like activity. The drug which has been shown to have such activity but is not structurally similar to T3 or T4 is amiodarone.

Amiodarone is a benzofuran having a chemical formula (2-butyl-3-[3,5-diiodo-4-(β-diethylaminoethoxy)-benzoyl] benzofuran). Amiodarone is a potent drug widely used for

the treatment of angina pectoris, ventricular and supraventricular arrhythmias, which has a number of effects on parameters of thyroid function. For example, chronic administration of amiodarone has been associated with both hyper- and hypothyroid-like side effects. Clin. Endocr., 22:257 (1985). The drug has also been reported to cause changes in the concentrations of serum thyroxine (T4) and triiodothyronine (T3) levels, which have been attributed to an inhibition of peripheral T4 monodeiodination, and to iodine-induced changes in glandular hormonogenesis. J. Clin. Invest., 58:255 (1976). However, cases of clinical hypothyroidism have occurred, often with mildly elevated thyroid-stimulating hormone (TSH) and normal or slightly decreased T4 and T3 serum levels, which produce decreased pituitary thyroid receptor hormone binding, see Clin. Endocr., 22:257 (1985).

Despite the chemical and structural dissimilarities, these observations suggest that amiodarone could act as a thyroid hormone antagonist at the receptor level. If that is true, then other structurally dissimilar compounds and drugs could also possess such ability but because of their obvious chemical dissimilarity, such pharmacological ability would seldom or never be discovered.

Thus it would be very advantageous to have available method which would quickly and effectively determine whether the compound does/does not possess a pharmacological activity and whether it would act as either the agonist or

the antagonist on the receptor level.

Despite the research in this area, a predictive method to select novel pharmacologically active compounds, e.g. 4-substituted and hydroxyl substituted derivatives of 2,6-diiodophenol, having useful pharmacological action in a mammal, or discovering new utilities for known compounds has not been presented. In addition, the compounds potentially predicted as having useful pharmaceutical activity may95Xnot have been disclosed or prepared.

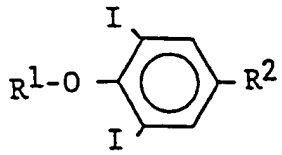
The present invention provides such a predictive method and the compounds having a useful pharmaceutical activity in a mammal.

All references and documents cited herein are incorporated in their entirety by reference.

SUMMARY OF THE INVENTION

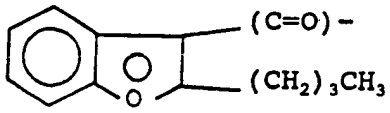
In the first aspect, the present invention relates to method of predicting and determining pharmacological activity of compounds having certain spatial atom arrangements.

In the other aspect, the present invention relates to a method for selecting compounds having useful pharmacological properties in a mammal from the group of compounds of the structure:

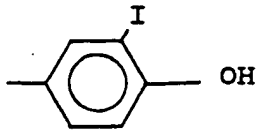


(1)

wherein R^1 and R^2 are each independently selected from aliphatic moieties, substituted aliphatic moieties, aromatic moieties or substituted aromatic moieties with the proviso that R^1 and R^2 are not both unsubstituted methyl or ethyl or a combination thereof, and when R^1 is $-CH_2CH_2N(CH_2CH_3)_2$, or $-CH_2CH_2NHCH_2CH_3$, R^2 is not,



or when R^1 is



R^2 is not $HOC(=O)CH(NH_2)CH_2-$,

which method comprises orienting the compound of structure (1) into a conventional cartesian three dimensional x, y, z coordinate space axes of Figure 3 in a manner such that the plane of the phenyl/iodine atoms is in the x, y plane, the minus y-axis bisects the phenyl ring, and the carbon atom in the phenyl ring in the para-position to the attached oxygen atom of T-3 is fixed on the 0,0,0-coordinate having standard distances between atoms based on the carbon-carbon single bond of 1.54 angstroms, and assess the volume spatial characteristics according to th

following three dimensional space shown in Figures 4A and 4B;

wherein R^1 is spatially within space A which is defined as between +3.5 and -3.5 angstroms on the x-axis, between -5.1 and -16 angstroms on the y-axis, and between +3 and -8 angstroms on the z-axis; and

R^2 has van der Waals spatial characteristics comprising spaces B^1 and B^2;

wherein B^{1a} is the space within -4.3 to +5.2 angstroms on the x-axis, +4.5 to +8.5 angstroms on the y-axis, and -3.5 to +3.5 angstroms on the z-axis; and B^{1b} is the space within 0 and -5.0 angstroms on the x-axis, -5.1 and +4.5 angstroms on the y-axis and 4 and -3 angstroms on the z-axis, and B^{1c} is defined as the space within 0 and 6 angstroms on the x-axis, -5.1 and 4.5 angstroms on the y-axis and 0 and -3 angstroms on the z-axis, and

wherein B^2 is between 0 and +10 angstroms on the x-axis, between -5.1 and +4.5 angstroms on the y-axis and between 0 and +10 angstroms on the z-axis.

Another aspect of this invention utilizes the commercially available computer programs in preparing three dimensional spatial models of investigated molecules, which allow the superimposing of these models over the model of the standard molecule and the rotational orientation of the

3-dimensional structure for selection of pharmacologically active compounds.

In another aspect, in Compounds of structure (1),

R^1 is selected from:

(a) $-CH_2CH_2N(R^4)R^5$ wherein R^4 and R^5 are the same and are selected from $-CH_2CH_3$, $-CH_2CH_2CH_3$, $-CH_2CH_2CH_2CH_3$, $-CH_2CH(CH_3)CH_3$, $-CH_2CH_2OH$, or $-CH_2CH_2CH_2OH$; or

R^4 is methyl and R^5 is selected from $-(CH_2)_4OH$, $-(CH_2)_5OH$, $-(CH_2)_6CH_3$, $-CH_2CH(CH_3)_2$, $-CH_2$-phenyl; or

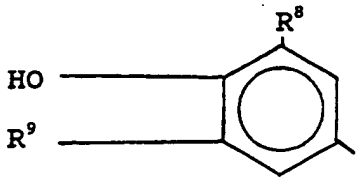
R^4 and R^5 together form $-(CH_2)_5-$, or $-CH_2CH_2OCH_2CH_2-$; or

(b) $-CH_2CH_2CH_2-N(R^6)R^7$ wherein:

R^6 and R^7 are the same and are selected from $-CH_3$, $-CH_2CH_3$, or $-(CH_2)_2OH$; or

R^6 is methyl and R^7 is selected from $-CH_2CH_3$, $-CH_2CH_2CH_3$, $-CH_2$-phenyl, $-CH_2CH_2OH$, or $-CH_2CH_2CH_2OH$; or

(c)



wherein:

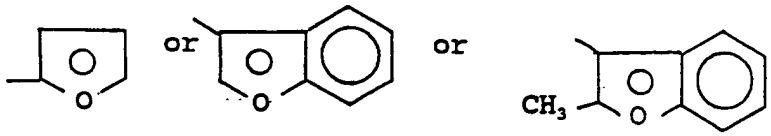
R^8 and R^9 are both $-CH(CH_3)_2$, or R^8 is -H, and R^9 is $-(CH_2)_5OH$, or R^8 is -H and R^9 is -I; and

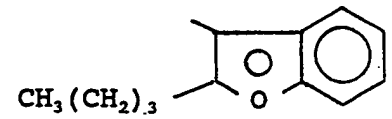
wherein: R^2 is selected from

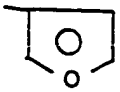
(a') $-(CH_2)_n-\overset{O}{\overset{\|}{C}}-R^{10}$

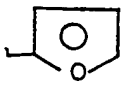
wherein n is 0, 1 or 2, and

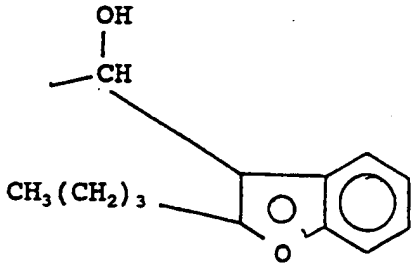
wherein when n is 0, R^{10} is -OH, or $-CH_3$,

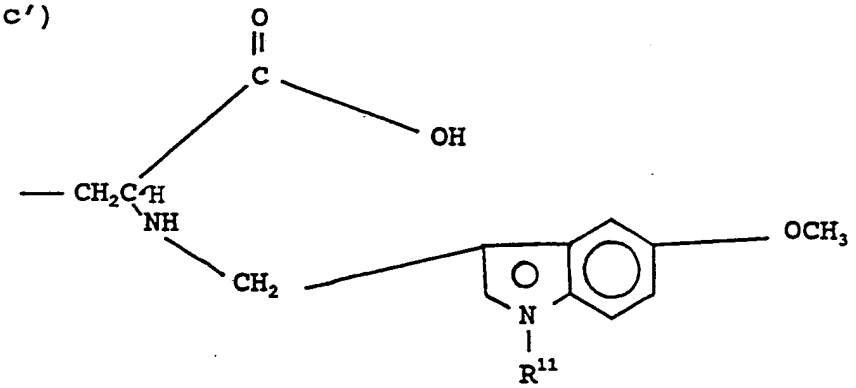


or  ;and

wherein when n is 1, R^{10} is -OH, or  ;and

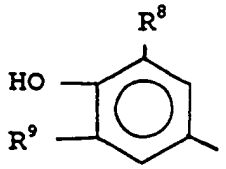
wherein when n is 2, R^{10} is  ;or

(b′)  ; or

(c′) 

wherein R^{11} is selected from -H, -(C=O)-phenyl, or para-(CH_3O)-phenyl-;

with the proviso that when R^1 is



and R^2 is (a') when n is 0 or 1, then R^{10} is not OH.

In a preferred embodiment only when the test compound has substituents in the predetermined volumes, a binding evaluation is undertaken. Only when the binding assay is positive, the experimental compound is recommended for further pharmacological evaluation.

In a preferred embodiment R^2 has at least four atoms, preferably selected from carbon, oxygen, nitrogen or combinations thereof.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows computer-generated models of T3 and amiodarone in a nondeformed state.

Figure (1A) shows van der Waals forces associated with the iodine atoms illustrated for each molecule. Triodothyromine T3 is on the left, amiodarone on the right.

Figure 1(B) shows hypothetical alignment of molecules along their respective "vertical" axes.

Figure 1(C) shows superimposition of di-iodo phenyl rings showing similarity of space filling by surface van der Waals forces.

Figure 1(D), shows superimposition of di-iodo phenyl rings showing similarity of space filling by surface van der Waals forces, with 90°C rotation along vertical axes. Slight changes in amiodarone torsional angles increases the similarity of the superimposition of amiodarone and the "lower" outer ring of triiodothyronine.

Figure 2 shows two chemical structures. Figure 2A shows amiodarone structure. Figure 2B shows trioodothyronine structure, also designated T3.

Figure 3 depicts a conventional cartesian three dimensional x, y, z coordinate space axes and the orientation of structure (1).

Figures 4A and 4B are the spatial models of three dimensional x, y, z coordinate space axes depicting space A and space B as projected on the y-z and x-y planes where R^1 substituent of the compound (1) is spatially within space A and R^2 substituent of the compound (1) is within space B.

Figure 5 illustrates competition by amiodarone and T3 for binding of $L^{125}I$-T3 to nuclear thyroid hormone receptors from different tissues.

Figure 6 (as 6A and 6B) illustrates the competitive nature of the effects of amiodarone on saturation binding of T3 to soluble nuclear thyroid hormone receptors.

Figure 7 (as 7A and 7B) shows effects of amiodarone on accumulation of rGH mRNA and on binding by T3 to nuclear

fractions of cultured GC cells.

Figure 8 depicts receptor binding of T3 and amiodarone.

Figure 9 depicts receptor binding of T3 and of the experimental compound (1) wherein R^1 is as shown in Table 1 where R^4 and R^5 are both 1a, and R^2 is 3c, as shown in Table 2.

Figure 10 depicts a receptorbinding of T3 and of the experimental compound (1) wherein R^1 is as shown in Table 1 where R^4 and R^5 are both 1a, and R^2 is 8c, as shown in Table 2.

Figure 11 depicts receptor binding of T3 and of the experimental compound (1) wherein R^1 is as shown in Table 1 wherein R^4 and R^5 are both 1a, and R^2 is 10c, as shown in Table 2.

Figure 12 depicts receptor binding of T3 and of the experimental compound (1) wherein R^1 is 24a, as shown in Table 1, and R^2 is 13c, as shown in Table 2.

Figure 13 depicts a receptor binding of T3 and of the experimental compound (1) wherein R^1 is 24a, as shown in Table 1 and R^2 is 15c, as shown in Table 2.

DETAILED DESCRIPTION OF THE INVENTION

This invention concerns a novel method of predicting, evaluating, and designing pharmacological activity of the experimental compounds on the basis of their spatial atom arrangements. Three dimensional spatial model has been developed based on conventional cartesian three dimensional x, y, z coordinate space axes within which the structure of

standard compound, having the predetermined desirable pharmacological activity, is entered by using conformation van der Waals characteristics. The three dimensional structure model of the experimental compound of which the pharmacological activity is evaluated, with respect to the mimicking of the pharmacological activity of the standard compound, is superimposed on the three dimensional spatial model of the standard compound in a manner similar to that of the standard compound, and the structure of the experimental compound is oriented by rotation on x, y, z plane to become spatially as close as possible to that of the standard compound. This is achieved by superimposing the same segment of the experimental and standard molecule and by rotating each substituent of the experimental molecule in such a manner that it fits within the certain space which has defined x, y, z coordinates on both plus (+) and minus (-) sides of the x, y, z axes plane, as shown in Figures 3 and Figures 4A and 4B, within which each substituent of the standard compound is located and positioned.

The first precondition for the experimental compound to have the same or similar pharmacological activity of the standard compound (e.g., Structure 1) is that the substituents of the experimental compound must fit within the predefined volumes and spaces within which the substituents of the standard compound fit. If the two or more remotely located substituents, for example R^1 and R^2, of

the experimental compound fit in the same spaces as substituents of the standard compound that is in space A, space B^{1b}, B^{1c} and B^{1a} and a small portion of less than 10%-20% fits in space B^2, there is a good chance that the experimental compound will have the same or similar pharmacological activity, that is, that it is an agonist acting on the same receptors as the standard compound. On the other hand, if only one substituent of the experimental compound is located within the space defined for and usually occupied by the substituent of the standard compound and additional volume of the other substituent is occupied by the volumes defined in B^1 and B^2, with the other substituent occupying greater than 10-20% of space B^2, then the pharmacological activity of the experimental compound is likely to be antagonistic, i.e. acting, for example, as an inhibitor of the standard compound.

By the way of the example, this invention is practiced by using as a standard compound a thyroid hormone T3, having a well defined and specific regulatory and metabolic agonist function on the thyroid cell receptors, and by using as an experimental compound the known antiarrhythmic drug amiodarone. Structurally, these two compounds, i.e. standard compound T3 and experimental compound amiodarone, differ. Except of the 2,6-diiodophenyl, which is present in both compounds, their substituents, as shown in Figure 2, are different. When however, the diiodophenyls of both compounds are superimposed and fitted into the three-dimensional model of this invention, their similarity is easily ascertainable, as seen in Figure 1D. Angstrom atomic

coordinates of both compounds using the defined 0,0,0 point are shown in Tables 1A and 1B.

TABLE 1A

T3

Å Coordinates for Atoms ± .25 Å

		X	Y	Z
C1		0	0	0
C2		-1.17	-.8	0
C3		-1.17	-2.1	-.1
C4		0	-2.8	-.2
C5		1.24	-2.1	-.2
C6		1.24	-.8	-.1
I-5		3.1	-3	-.4
O-4	(1)	0	-4.2	-.27
C1′		0	-4.9	-1.5
C2′		.42	-6.2	-1.4
C3′		.4	-6.9	-2.6
C4′		.15	-6.3	-3.9
C5′		-.3	-5	-3.9
C6′		-.4	-4.3	-2.7
O-4′	(2)	.2	-7.1	-5.0
I3′		.8	-9.	-2.6
I3		-3	-3.0	-.3
C-7		0	1.5	-0
C-8		1.3	2.1	+.5
C-9		1.3	3.6	.35
O-10	Carbonyl	.8	4.1	-.6

TABLE 1A (Continued)

O-9	1.9	4.3	1.2
N-8	1.4	1.8	1.9

1 Ring Bridge Oxygen

2 OH Oxygen

TABLE 1B

Amiodarone

Angstrom Coordinates for Atoms ± .25 Å

		X	Y	Z
C1		1.5	6.1	.6
C6		2.3	5.3	1.2
C5		2.0	4.0	1.1
O-7	Furan	2.8	3	1.66
C8		2.0	1.9	1.4
C10		2.4	.6	2.1
C11		3.0	.8	3.5
C12		3.4	-.5	4.2
C13		3.9	-.3	5.6
C9		.9	2.2	.6
C4		.9	3.5	.5
C3		.1	4.4	0
C2		.4	5.7	0
C14		-0.1	1.4	.2
O-15	Carb	-1.1	1.9	0
C-16		0	0	0
C-21		-1.1	-7	0
C-20		-1.1	-2.1	-.14
C-19		0	-2.8	-.25
C-18		+1.1	-2.0	-.25
C-17		+1.1	-.7	-.1
I11		2.9	-3.0	-.5
O-22	Ring	.2	-4.1	-.35
C-25		-.3	-5	-1.4

TABLE 1B (Continued)

Amiodarone

Angstrom Coordinates for Atoms ± .25 Å

	X	Y	Z
C-26	.2	-6.4	-1.1
N-27	-.2	-7.4	-2.1
C-30	+.3	-7.1	-3.5
C-31	0	-5.7	-4.0
C-28	+.2	-8.8	-1.6
C-29	-.3	-9.2	-.34
I-10	-3.0	-3.2	-.22

Once such structural similarity is prove, the pharmacological activity, whether agonist or antagonist, is almost certainly present. Such activity is then quickly determined by the receptor binding assay. Only when both the structural spatial similarity exist and the binding to the receptor is positive, the compound is suitable for broad pharmacological testing with reasonable probability that either agonist or antagonist activity of the drug exist, making it worthwhile for extensive pharmacological testing and development into a pharmacological drug.

Since the broad range of pharmacological tests are generally necessary to determine whether or not there is or there is not a pharmacological activity present, the current invention represent a substantial savings of the resources and money. In view of the thousands of newly synthesized compounds of which pharmacological activity must be determined, the magnitude of savings achieved by this

invention is easily apparent. By simply using two tests, one computerized, the second simple receptor binding assay in the laboratory, the need for further pharmacological testing is easily confirmed or disproved.

Moreover, due to a seeming one or two dimensional structural dissimilarity, many compounds would never even be tested for certain pharmacological activity since such activity would not be expected from their one or wo dimensional chemical structures.

Consequently, the current invention also provides for discovery of new pharmacological activities for compounds where it would not be expected or suspected, and therefore would not be tested for.

Finally, the current invention is useful in predicting and designing new compounds based on pharmacological activity of known compounds. By way of example, when compound X is known to have certain desirable pharmacological activity, but its substituents confers on it rather undesirable side effects, the practice nowadays is that the closest possible structures are designed and the substituents responsible for the side effects are changed. Each new derivative is tested, until the undesirable properties are either eliminated or decreased to the acceptable level. By utilizing the current invention, the compound which is not necessarily structurally similar but which fits the spatial model of this invention may be designed by combining the molecular components responsible

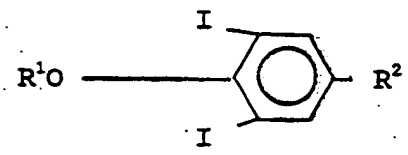
only for desirable pharmacological properties, regardless whether these components combined together are structurally similar or not.

The current invention thus provides fast, easy, and reliable method for evaluation of pharmacological activity of large number of compounds which method eliminates unnecessary laboratory testing which is costly, laborious and slow and which is currently based primarily on the structural similarity to standard compound of which pharmacological activity is the new compound supposed to mimic, substitute or countermand.

PREFERRED EMBODIMENTS

Preferred embodiments of this invention are compounds which fits within three dimensional spatial model of the standard compound having certain predetermined pharmacological activity.

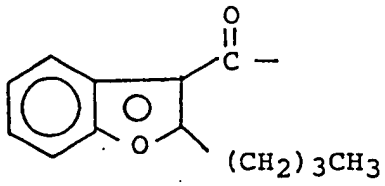
More preferred embodiments of this invention are compounds chosen by the method of this invention from the group of compounds of structure:



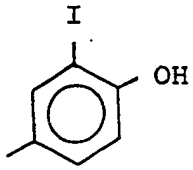
(1)

wherein R^1 and R^2 are each independently selected from aliphatic, substituted aliphatic, aromatic or substituted aromatic moieties with the proviso that R^1 and R^2 are not both at the same time unsubstituted methyl or ethyl or

combination of both and when R^1 is $-CH_2CH_2N(CH_2CH_3)_2$, or $-CH_2CH_2NHCH_2CH_3$, R^2 is not



or when R^1 is



R^2 is not $HOC(=O)CH(NH_2)CH_2-$.

More preferred embodiments are the compounds chosen by the method of this invention wherein the three dimensional model of the standard and experimental compounds are superimposed over each other and the spatial similarity is found suggesting pharmacological activity which is confirmed by receptor binding assay.

DEFINITIONS

"Aliphatic moiety" refers to alkyl, alkenyl, acetylenyl, cyclic, acyclic and the like compound having between about 1 to 20 carbon atoms. Acids, ketones, aldehydes, alcohols, sulfides, amines, imines, and the like and combinations thereof are found within the aliphatic groups. Preferably the aliphatic moiety has about 1 to 10 carbon atoms.

"Aromatic moiety" refers to a cyclic compound which has from about 5 to 25 carbon atoms, having conventional aromatic properties. Compounds include benzene, toluene, naphthalene, hexane, heptane and the like. Heterocyclic compounds (groups) may have one or more carbon atoms in a ring replaced by at least one polar atom such as oxygen, nitrogen, sulfur and the like. Combinations of hetero atoms are contemplated.

"Substituted aromatic moiety" refers to aromatic groups as defined herein wherein at least one ring proton is substituted by a group such as hydroxy, chloro, bromo, iodo, amino, -SH, -COOH, -C=O, CH(=O), and the like.

"Pharmaceutically acceptable acid addition salt and ester" refers to those salts which retain the biological effectiveness and properties of the free bases and which are not biologically or otherwise undesirable, formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ehtanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like. The pharmaceutically acceptable acid addition salts and esters are intended to be included in the present invention of Structure (1).

"Substituted aliphatic moiety" refers to aliphatic moiety which has one or more protons substituted by a polar atom or combinations thereof selected from oxygen, nitrogen, sulfur.

"Standard compound" means any pharmacologically active compound of which three dimensional spatial atom arrangement can be made into three dimensional computerized model using the van der Waals characteristics.

"Experimental compound" means any compound of which three dimensional spatial atom arrangement can be made into three dimensional computerized model using the van der Waals characteristics.

Amiodarone biologically fulfills the criteria of a thyroid hormone antagonist. The drug behaves as a competitive inhibitor of triiodothyronine (T3), binds to solubilized thyroid hormone receptors, and blocks both receptor binding and the biological effect of thyroid hormone when administered to hormone-responsive cells in culture. J. Clin. Invest., 83: (1989).

Structure-activity studies have suggested the necessity of bulky iodine or propyl substituents on the outer ring 3' position and the presence of a carboxylic acid group for effective receptor binding. Amiodarone possesses neither of these moieties, and indeed comparison of the two-dimensional chemical structures reveals no obvious structural similarity between the drugs.

The biological activity which points toward antagonistic activity of amiodarone to thyroid hormone is in direct disagreement with its structure. As seen in Figure 2A depicting amiodarone and in Figure 2B depicting T3, while their molecular weight of M.W. 645 and M.W. 651, respectively, is fairly close, their chemical structural formula is very dissimilar. In fact, the only identical structural component present in both compounds is the aromatic moiety 2,5-disubstituted with iodine. Consequently, seemingly there is no structural similarity in one dimensional chemical structure.

Similarly, comparison of both structures two-dimensionally reveals no obvious structural similarity between both structures, since the amiodarone possesses no carboxylic iodine or propyl group substituents on the 3-position of the outer aromatic drug which is believed to be necessary for effective receptor binding and agonist function (End. Review, 1:140 (1980).

And yet, both compounds seem to interact with the same receptor. Thus, if there is some possible structural similarity between T3 and amiodarone, it must be in some spatial arrangement of atoms which assures the binding of amiodarone to the receptors identical to those which bind to thyroid hormone. The Figure 1A shows van der Waals forces which are associated with iodine atoms substituting the aromatic ring 2,5-positions in both T3 and amiodarone molecules. When the both molecules were aligned,

hypothetically, along their respective vertical axes, as shown in Figure 1B, the only points of similarity are the aromatic rings with two iodines sticking out, and a partially similar aliphatic chain parallel to each other in position 1 and 4. There the similarity ends.

When, however, as shown in Figure 1C both molecules were superimposed over their di-iodo phenyl rings, they showed similarity of space filing by surface van der Waals forces and when, as in Figure 1 D, these structures were rotated 90° along their respective x, y and z axes, the similarity was readily visible, particularly the similarity of the R^1 substituent of amiodarone and the lower outer ring of T3. This similarity may be increased by slight changes in amiodarone torsional angles.

If such biological-spatial relationship exists between T3 and amiodarone, they exist presumably also for other seemingly structurally unrelated molecules and it is therefore a primary object of this invention: (a) to provide a method for testing currently known molecules having nonsimilar structures to the endogenous or synthetic known agonists or antagonists, (b) to design new molecules having the similar superimposable structural similarities and characteristics and (c) to provide a fast and efficient method for testing of the new and known molecules by using the system of this invention combined with the biological evaluation of the molecules, namely combined with binding r ceptor assay used to determine quickly whether spatial

arrangements of the molecules has the expected biological activity.

Determination of Spatial Atom Arrangements

The spatial atom arrangements of the molecules which are candidates to be used in practice of this invention, are determined by choosing a standard molecule which is either endogenous hormone or neurotransmitter, or synthetic pharmaceutical drug which acts as an agonists or antagonist in certain biological set-up, and is pharmacologically active on certain receptors. Then, a extended conformation molecular model, using standard data sets, of the standard molecule is constructed. The molecular model is constructed with the MIDAS program developed and available from the Computer Graphics Laboratory, University of California, San Francisco, CA. The model is based on using a standard data set and refined using a program such as MM2.

Using the MIDAS program, computer generated van der Waals surfaces are displayed around the various atoms forming the standard molecule. The model of the standard molecule is then oriented into a conventional cartesian three dimensional x, y, z coordinated space axes, described in Advanced Physical Chemistry, McMillan Co., UK (1969), in a certain manner which is specific to each molecule but which orients on the x, y, z axes. The portion of the molecule which is important functionally and substituents which may or may not be functionally important, as determined from the standard compound, are then superimposed over the same components of the standard molecule.

The molecular substituents are then individually determined to be within certain space or volume which has be n herein designated to be space A for substituents R^1 which is defined, in angstroms, as a distance in plus and minus angstroms on the x, y or z axes, or as a space between plus and minus places, expressed in angstrom, on the x, y, or z axes shown in Figure 3. Similarly, substituent R^2 is determined to be spatially within certain spaces and volumes, herein called space B, again expressed in distances in plus and minus in angstrom on x, y, and z-axis. In similar manner, all substituents of the standard molecule are placed in certain determined space defined on axes x, y and z in minus and plus space of each axis. The spaces A and B are illustrated in Figure 4A and 4B. In this particular case, the space A determines and limits spatial atom arrangement of substituent R^1, and space B determines atom arrangement of the substituent R^2.

When the spatial model for standard active molecule is prepared, using the same procedure, the model of the second experimental molecule is then prepared in the same way.

If the experimental molecule is a known chemical which has seemingly different chemical structure but similar biological activity, both models are superimposed over each other and the structural spatial similarity is determined. If such spatial similarity exists, combined with its biological activity, then the prove is obtained that the xperimental molecule is binding to the same receptor which also binds the standard molecule.

If the chemical structure of the experimental molecule is not known and the new molecule is to be designed having similar biological and pharmacological activity, than the model of the standard molecule is used to design a number of structurally similar compounds, in spatial arrangement, similar to the standard compound. The model of the designed compound is then superimposed over the model of the standard compound and those compounds which are spatially similar to standard compound are tested for their biological activity.

Thus, using the procedure of this invention, a large number of compounds may be quickly and easily examined and the expensive and lengthy testing avoided. Moreover, the need for actual synthesis of many compounds is effectively eliminated because only those compounds which are spatially similar to the standard compound, will be synthesized and tested pharmacologically.

The commercial computers used in this invention are "VAX", Digital Equipment, Maynard, Massachusetts, using publicly available "VMS" and "UNIX" operating systems.

The Quantum Chemistry Program Exchange (QCPE), Department of Chemistry, Indiana University, Bloomington, Indiana, publicly available computer program "MM2" is used to refine the coordinates for amiodarone and T3, or alternatively the T3 coordinates are extracted from the publicly available Cambridge Crystallographic Structural Data Base, Cambridge University, Cambridge, England.

Commercially available drug design computer programs of interest also include, for example, program "SYBYL", available from Tripos Associates, St. Louis, Missouri; or "BIOGRAF", from Biodesign, Inc., Pasadena, California.

The interatomic distances for benzene or other C=C, C-N, C-O, C=O, etc. are found in Chemistry of Organic Compounds, 2nd Ed., C.R. Noller, W. B. Sanders Company Philadelphia, Pennsylvania (1957).

For Computer Modeling, the VAX computer is loaded with VMS or UNIX operating computer system, commercially available.

The coordinates for the atoms of amiodarone from a standard data set are input into the VAX computer program and refined by using MM2 program.

The coordinates for the atoms of T3 are input into the VAX computer program system.

Additionally, the "BIOGRAF" program may be added to the VAX program system.

By manipulating the computer program of the spatial structural atoms of amiodarone and T3, as shown in Figure 1B, it is apparent that the diiodophenol rings in each are essentially superimposable.

From this spatial relationship, the surprising essential spatial features of pharmacologically active molecule is obtained. These spatial features then are useful to select compounds which have useful pharmacological properties and may interact with the receptors in the cells of a human

being to mitigate, alter, inhibit or otherwise affect the disease conditions described herein.

According to this method, it is observed that both T3 and amiodarone occupy R^1 space A but that amiodarone significantly contributes to space B^2 much of which is not occupied by the T3 molecule. Substitutions of N-acyl groups in a portion of this area, described in Biochemitry, 21:163 (1982), led to diminished binding and agonist activity. Amiodarone does not contain the polar N-acyl groups but instead uses a non-polar and flexible hydrocarbon moiety.

This observation suggests that substitutions containing alkyl groups in B^2 space but without N-acyl moieties, to T3 or other thyroid hormones analogs such as thyroid hormone-acetic acid, might product antagonist activity.

In defining R^1 and R^2 groups for spatial characteristics, the groups are predominantly (substantially) within space A for R^1 and B^1 and B^2 for R^2. At least 90% of the structure is within these spaces. Space filling of B^2 with atoms of the structures described herein is preferred.

From the conformation studies, it is assumed that either T3 conformation is not markedly deformed by the receptor in the process of receptor binding or that both T3 and amiodarone or other antagonist and derivatives thereof are sufficiently flexible to allow similar degrees of deformation. Spaces B^1 and B^2 could conceivably occur in the mirror image location via rotation, e.g. 180°, around the C-C bond between the ketone and phenyl moiety located in

space B between 0, 0, 0 (x, y, z) and approximately 0, 1.5, 0 angstrom. This conformation could equally occur in T3. Consequently the space B can be as shown or in its rotational image space $B^{2'}$.

In a preferred embodiment, for this particular pair of compounds, the coordinates for R^1 (space A) are between about 3 and -3 angstroms on the x-axis, -2.5 to -12 angstroms on the y-axis, and +2.0 to -7 angstroms on the z-axis, and for R^2 space B^1 and B^2 where R^2 has van der Waals spatial characteristics comprising spaces B^1 and B^2. B^1 is the area falling outside B^2. In the case of T3 and amiodarone examples shown in Figures 1C and 1D, B^2 is defined by two sets of coordinates:

B^{1a} is defined as the space within -4.3 to +5.2 angstroms on the x-axis, +4.5 to +8.5 angstroms on the y-axis, and -3.5 to +3.5 angstroms on the z-axis, and B^{1b} is defined as the space within 0 and -5.0 angstroms on the x-axis, -5.1 and +4.5 angstroms on the y-axis and 4 and -3 angstroms on the z-axis, and B^{1c} is defined as the space within 0 and 6 angstroms on the x-axis, -5.1 and 4.5 angstroms on the y-axis and 0 and -3 angstroms on the z-axis, and

B^2 is between about 0 to +8 angstroms on the x-axis, +3.1 to -2.8 angstroms on the y-axis, and between 0 and +8 angstroms on the z-axis.

Since the amiodarone was already shown to have all properties needed in practicing this invention, it was used as the model of experimental compound.

The cartesian rectangular coordinates as described herein are found in Advanced Physical Chemistry by S.M. Blinder, published in 1969 by the MacMillan Co., Collier-MacMillan, Ltd. of London, United Kingdom.

To explore a possible structural similarity between T3 and amiodarone, extended conformation molecular models, as shown in Figure 1 using standard data sets, were constructed with the MIDAS program as described above. XFig. 1A displays triiodothyronine (left) and amiodarone (right), each with computer-generated van der Waals surfaces displayed around their respective iodine atoms. When the diiodopheyl rings of each compound were juxtaposed a remarkable degree of similarity can be seen along their entire vertical axes (Figure 1B). Complete superimposition of the diiodophenyl rings leads to the structures shown in Figure 1C and, rotated 90° along their "vertical" axes, Figure 1D. A striking homology exists between the van der Waals surfaces of each drug use. The lower portion of the superimposed structures containing the inner and outer rings of triiodothyronine displays the greatest degree of similarity. This portion of the T3 has been proposed to interact with the receptor protein. J. Med. Chem., 20:863 (1977) This portion of amiodarone is more flexible than the outer ring of T3. However, the upper portion of the structures, containing benzofuran and its 2-butyl adduct, is most dissimilar, suggesting a structural basis for antagonism. Most strikingly, the butyl group lies spatially in an area

completely vacant from T3 occupation. This may account for certain antagonist properties of amiodarone. However, amiodarone has limited utility as a thyroid hormone antagonist because of certain properties. These properties are the drug's low affinity for the thyroid hormone receptor, probably due to the spatial dissimilarity of its lower portion in space A, numerous amiodarone-induced toxic side effects, and reported amiodarone effcts that are not related to thyroid hormone activity, such as for example, amiodarone's inhibition of in vitro phospholipase A_1 production in rat alveolar macrophages, J-774 macrophages and rat liver when used at concentrations 5-10 times greater than that required to inhibit T3 receptor binding by 50%. Biochem. Biophys. Acta., 875:400 (1986). Finally, the drug has a long half-life and accumulates extensively in tissues which could hinder the ability to regulate the actions of the drug.

Nevertheless, the present invention demonstrates that amiodarone interacts directly albeit with low affinity, with the nuclear thyroid hormone receptor, and that it antagonizes the effect of hormone on the production of hormonally induced rat growth hormone RNA. In addition, it indicates that other amiodarone-like (e.g., 4-substituted, HO- or O-substituted 2,6-diiodophenol) derivatives have the potential to be of considerable practical value both clinically as cardiotherapeutic drugs and as tools for further investigating the molecular mechanisms of thyroid

hormone action, as is disclosed in the present invention.

The starting materials used for the compounds are for the most part commercially available from the listing of chemical supply houses published annually by Chemical Sources, U.S.A., Columbia, South Carolina, or in the Merck Index published annually by Merck & Co., Rahway, New Jersey. Sigma, St. Louis, Missouri and Aldrich Chemical Company, Milwaukee, Wisconsin, are the usual commercial sources.

The method used in the preparation outlined briefly here are well known in the art and described in publicly available chemical handbooks, textbooks and other materials.

The N-alkyl compounds of triiodothyronine are described more specifically in the Examples. One useful approach is to protect the carboxyl group of triiodothyronine by conversion to an ester using an alcohol (e.g. butyl) and trifluoroacetic anhydride as a catalyst. The ester is then treated with an alkyl halide (e.g. butyl bromide) and recovered. The ester is then converted to the carboxylic acid T3-derivative, using techniques such as treatment with base, such as 1-N sodium hydroxide.

UTILITY

This invention is useful for fast, efficient, easy and economical evaluation of pharmacological properties of drugs having certain three dimensional structural atom arrangement. When the spatial arrangement is similar to that of a known pharmacologically active compound and when the pharmacological activity can be confirmed by simple

laboratory assay, the probability is high that such compound will have the same agonistic, or contrary antagonistic activity.

A preferred pharmaceutical use of the compounds selected by this method is in the treatment of thyroid hormone disorders such as hyperthyroidism and hypothyroidism.

The useful pharmacological properties are selected to treat a condition of hyperthyroidism, angina pectoris, antiadrenergism, cardiac arrythmia, cardiac ischemia or neurologic and psychiatric disorders and conditions in a human being.

The following examples are meant to be explanatory and illustrative only and are not to be construed as being limiting in any way.

EXAMPLE 1

N-NORMAL-BUTYL TRIIODOTHYRONINE

(a) Commercially available triidothyronine (1 eq.) is combined with normal butyl alcohol (1.1 eq.) in the presence of a trace of an esterification catalyst trifluoroacetic anhydride, in 200 ml of solvent of dimethylformamide. The reaction mixture is held at ambient temperature overnight and then carefully heated to 50°C for one hour. The butyl carboxylic ester is obtained after a careful aqueous work up using brine. The phenol and amine functional groups increase solubility in water.

(b) The carboxylic ester obtained is then dissolved in about 500 ml of dimethyl formamide. To this solution is

added at ambient temperature dropwise butyl bromide (1 eq.) in an equivalent volume of dimethyl formamide. After butyl bromide is added, the reaction mixture is held at ambient temperature overnight (20 hr.) and then heated to 50°C for 1 hour. The reaction mixture is contacted with water/ice and the organic portion is removed using methylene chloride. The methylene chloride is removed under vacuum. The butyl carboxylate ester-normal-butyl amine, is separated, recovered and purified using column chromatography-hexane/chloroform (50/50) silica gel from any homologs formed.

(b') when a 2.5 or greater excess of butyl bromide is used in step (b), the dibutyl amine derivative is a product and is isolated using column chromatography.

(c) The butyl carboxylate ester normal-butyl amine of step 1(b) above (0.1 eq.) is then dissolved in dimethylformamide and sodium hydroxide (1-N) is added (0.01 eq.) at ambient temperature. After addition, the reaction mixture is held at ambient temperature (20 hr.) and then heated to 40°C for 1 hr. The carboxylic acid normal butyl amine derivative is obtained by extraction using hexane/chloroform and purified using column chromatography silica gel (hexane/chloroform, 50/50).

EXAMPLE 2

N-ISOBUTYL TRIIODOTHYRONINE

Example 1 is repeated except that in step (a) normal butyl alcohol is replaced with a stoichiometrically

equivalent amount of isobutyl alcohol, and in step (b) the normal butyl bromide is replaced with a stoichiometrically equivalent amount of isobutyl bromide. The N-isobutyl triiodothyronine is obtained.

EXAMPLE 2A

Example 1 is repeated except that in step (a) normal-butyl alcohol is replaced with a stoichiometrically equivalent amount of isobutyl alcohol, and in step (b) the normal butyl bromide is replaced with a stoichiometrically equivalent amount of isobutyl bromide. The N-isobutyl triiodothyronine is obtained.

EXAMPLE 3

Method for Selecting Compounds Expected to Have Pharmacological Activity

This example illustrates the method of evaluation and selection of compound and predicting their pharmacological activity based on their three dimensional spatial atom arrangement.

A. Three Dimensional Space Plane

Three dimensional x, y, z coordinate axes plane was designed, as shown in Figure 3, according to the procedure described in Advanced Physical Chemistry, Ed. S.M. Blinder, MacMillan Co., London, U.K. (1969).

B. Choice and Preparation of Standard and Experimental Compounds

Standard compound was chosen to be triiodothyronine (T3) as shown in Figure 2B.

The experimental compound was chosen to be benzofurane compound amiodarone as shown in Figure 2A. Amiodarone was chosen to prove the validity of the current invention because it is seemingly and visibly, not structurally similar to the standard compound T3 as seen by comparison of Figures 2A and 2B. By its pharmacological activity, however, there is some evidence pointing toward its possible T3 antagonist function.

C. Computer Modeling of Standard and Experimental Compounds

Commercially available computer VAX obtained from Digital Equipment, Maynard, Massachusetts, using VMS and UNIX operating systems and MM2 computer program obtained through the Quantum Chemistry Program Exchange, University of Indiana, Bloomington, Indiana was used to extract the coordinates of T3. These coordinates were confirmed by the Cambridge Crystallographic Structural Data Base, Medical Foundation of Buffalo, New York.

Drug design computer programs SYBYL, obtained from Tripolis Associates, St. Louis, Missouri, BIOGRAPH, obtained from Biodesign, Pasadena, California, and MIDAS computer program, obtained from the (Computer Graphics Laboratory of the University of California, San Francisco, CA), were also loaded on the VAX computer.

The coordinates of both T3 and amiodarone obtained either from crystallographic studies or by standard data sets were input into the VAX computer and energy refinements

were performed using the MM2 program. Computer modeling was performed at the Computer Graphics Laboratory of the University of California, San Francisco. The compounds were modelled in extended conformation using standard geometries. Coordinates were displayed and manipulated in real time with the MIDAS program on a Silicon Graphics Iris 80GT System, available from Silicon Graphics, Mt. View, CA.

First the three dimensional model of T3, using the available coordinates, was displayed on the screen, on which the model of amiodarone, using the available coordinates was superimposed. The structure model of amiodarone was then manipulated by manipulating its spatial structural atoms together with manipulating the spatial structural atoms of T3.

In the computer models, both T3 and amiodarone were displayed with their respective computer generated van der Waals surfaces displayed around individual atoms. Where the diiodphenyl rings of both molecules were juxtaposed, a remarkable degree of similarity has been seen along their entire vertical axes, as shown in Figure 1B. Complete superimposition of the diiodophenyl rings led to structures shown in Figure 1c. Only very small torsion angle adjustments were required in order to superimpose the molecular structures. When both models were rotated 360° through space along their vertical axes, a striking homology was found between the van der Waals surfaces of both compounds T3 and amiodarone as shown in Figure 1D (at 90°

rotation). The lower portion, i.e., on -y axis of the superimposed structures containing the inner and outer rings of T3 displays the greatest degree of similarity. This portion has been proposed (J. Med. Chem., 20:863 (1977)), to be responsible for T3 interaction with the receptor. On the other hand, the structural dissimilarity exist between the upper portion of the structures, containing benzofuran and its 2-butyl adduct. This dissimilarity points toward and provides basis for antagonistic properties of amiodarone.

These observations were confirmed by biological and pharmacological tests shown in Example 4.

EXAMPLE 4

Pharmacological Testing of Amiodarone for Its Antagonistic Activity

This example describes a series of biological and pharmacological tests performed to confirm the antagonistic activity of amiodarone which has been predicted by the computerized three dimensional structural examination of amiodarone spatial arrangement as described in Example 3.

Cell Culture utilized GC cells obtained from the cellular culture facility at UCSF. GC cells were plated at 2-4 x 10^6 cells/100 mm tissue culture disk obtained from Falcone, and were maintained at 37°C, in the mixture of O_2/CO_2 95/5%, in DMEM 21 medium containing 10% of fetal calf serum, obtained from J.R. Scientific, Woodland, California, to which 100/ml of penicillin and streptomycin, and 2 mM of glutamine were added.

Thyroid hormones T3 and thyroxine T4 were removed from fetal calf serum with AG 1-X8 ion exchange resin from Biorad, Hercules, California by the method described in Endocrinology, 105: 80 (1979). Before treatment, T4 and T3 concentrations were determined to be 1.9×10^{-7} and 1.9×10^{-9} M, respectively. Following the treatment, in 100% thyroid hormone depleted (stripped) serum, T4 and T3 were 1.7×10^{-8} and non-detectable (less than 300 pM), respectively. Actual concentrations in 2% media were therefore 1/50 of those values.

Solubilized T3 nuclear receptors were prepared from rat liver according to procedure described in J. Biol. Chem., Vol. 263, p. 9409-9417. Solubilized nuclear receptors from the whole rat brain were prepared by the method described in Endocrinology, 103:943 (1978). Solubilized receptors from GC cells were prepared as follows. Confluent 150 cm^2 culture plates were rinsed for 5 minutes with PBS at 37°C and scraped with a rubber policeman in 5 ml PBS at 4°C. the 1000 xg cell pellet was suspended in 10 ml of Solution 1 consisting of 20 mM KPO_4, 4mM EGTA, 4 mM $MgCl_2$, 0.25 M sucrose, 0.5% NP-40, 0.5 mM PMSF, and 0.1% of MTG, having pH 7.6 and put on ice for 3 minutes. Suspension was centrifuged and cell lysate was washed with Solution A minus NP-40, centrifuged again and the pellet was resuspended in 10 ml of Solution B consisting of 20 mM KPO_4, 10 mM EDTA, 2 mM $MgCl_2$ having pH 7.2. The nuclei obtained above were gently mixed and allowed to sit at 300 xg for 10 minutes and

there resulting chromatin pellet was washed twice in 10 ml of Solution C consisting of 10 mM KPO_4, 1 mM $MgCl_2$, 0.1% MTG having pH 7.2. The washed pellet was then solubilized in 2 ml of Solution D consisting of 10 mM Tris, 1 mM $MgCl_2$, 0.5 mM EDTA, 400 mM KCl, 10% glycerol, 0.1% MTG, having pH 7.6. The chromatin was then sonicated in Solution D 30 x 1 second pulses. After 30 minutes incubation on ice, polyethyleneimine (PEI) was added to a final concentration of 0.01% and the solution was stirred gently for 5 minutes. Following centrifugation at 15,000 x g for 15 minutes, the supernatant was adjusted with 3% PEG, 0.02% PEI and 20 mM KPO_4. After 20 minutes on ice, this suspension was centrifuged again at 15,000 x g for 30 minutes and aliguots were removed, frozen in liquid N_2 and stored at -70°C. Treatment with PEI was introduced to remove any contaminating DNA. Yield of the procedure was 7.5 pmol for 900 x 10^6 cells having specific activity of 3.1 pmol/mg of protein and containing 5000 receptor sites per cell.

For <u>in vitro binding assay</u>, thyroid hormone receptor preparations, as described above, were incubated with varying concentrations on nonradioactive T3 or amiodarone for 18 hours at 4°C in a buffer consisting of 20 mM KPO_4, 0.5 mM EDTA, 1 mM $MgCl_2$, 400 mM KCl, 8% glycerol and 0.1% MTG. Bound and free labeled $^{125}\Psi$I-T3 were separated with G-25 sephadex. Receptor concentrations were determined to be 150-300 pM. L-T3 was obtained from Aldrich Chemicals. A 1 mM stock solution was prepared in MeOH containing 1% NH_4OH.

This solution was diluted in 0.1 mM NaOH and added directly to binding and culture assays. Stock solutions of amiodarone-HCl, obtained Sanofi Chemicals, France, were prepared fresh at 14 mM in EtOH:10mM HCl (1:1). Dilutions were prepared in 1 mM HCL. L-^{125}I-T3 NEX-110X, 2200 Ci/mmol was obtained from New England Nuclear.

Binding of ^{125}I-T3 to nuclear receptors of intact GC cells was achieved by plating two million cells per well of G-well tissue culture plates of 9.3 cm^2/well in 3 ml of media. After 18 hours, cells were rinsed with 1 ml of PBS for 10 minutes at 37°C and incubated for four hours in DMEM containing 10% serum substitute solution and 2% stripped calf serum. For competition assays, cells were incubated in 1 ml of stripped media containing 300 pM of ^{125}I-T3 and various doses of amiodarone. Incubations were carried out at 37°C on a Tilt table. After 3 hours during which time equilibrium was established, cell nuclei were prepared by lysis in situ and specific nuclear binding determined according to established methods for pituitary tumor cells according to procedure described in Proc. Natl. Acad. Sci., 70:3488 (1973). Binding in each nuclear pellet was normalized to DNA content, which was determined according to Biochem. J., 62:315 (1956). Under these conditions maximum binding capacity was about 85 fmol/100 ug DNA. Relative to L-T3, no effect of amiodarone on cell surface transport of ^{125}I-T3 was observed.

Analysis of receptor binding parameters was done and binding data were analyzed with Scatfit and Allfit computer programs described in Am. J. Physiol., 235 E97-E102 (1978) and Molec. Pharm., 21:5-16 (1982). These programs perform non-linear, least squares model fitting utilizing untransformed data by sequential interaction of binding parameters and generate K_d, Bmax, pseudo-Hill slope constant (b) and EC_{50} parameters. The ability of amiodarone to compete with T3 for binding to the thyroid hormone receptor was assessed by comparison of K_d and EC_{50} values generated by the Scatfit and Allfit programs, respectively.

Preparation and Quantitation of Cytoplasmic RNA was done by plating and attachment for 12-18 hours of GC cells, followed by deinducing 4 days in 20 ml of DMEM containing 10% serum substitute solution obtained from Cell Culture Facility, UCSF, and 2% of stripped serum, prepared above. Media were replaced after the second day of deinduction. After 4 days of deinduction, cells were cultured for additional two days in stripped media containing either (1) 300 pM T3 plus one of the following dose of amiodarone: 0, 10 mM, 100mM, 1 uM or 10 uM; or (2) no additional treatment (NT). Total cytoplasmic mRNA was prepared according to Methods Enzymol., 65:718 (1980).

The amount of rGH message present in aliquots of GC cell cytoplasm was determined by Northern hybridization using glyoxylated RNA (Ibid., p. 380). Equal amounts of cytoplasmic RNA were examined per lane (15 or 20 ug). HeLa

cell RNA was also tested as a control for specificity and background binding. The rGH message was detected by hybridization with the 0.8 kb pRGH-1 rat growth hormone cDNA according to Nature, 270:486 (1977). Some blots were also probed with a 1.6 kb Kho I fragment of the pHF1 human gamma-actin gene according to Mol. Cel. Biol., 3: 787 (1983). Both probes wereXlabelled by nick translation with ^{32}P-dCTP, obtained from Amersham to 3-5x 10^8 cpm/ug according to J. Mol. Biol., 113:237 (1977). Hybridizations were carried out for 18-48 hours at 42°C. Blots were autoradiographed with Kodak XAR-2 film using Cronex Lightning plus intensifying screens.

The results of the studies are summarized in Table 1 and in Figure 5-7.

Using crude preparations of KCl-solubilized rat liver nuclei, T3 was shown to bind with high affinity to a single class of receptor cites, (K_d=359pM). This value was used was an initial estimate of receptor affinity for other studies.

TABLE 1C

Binding Affinities

Receptor Source	Triiodothyronine		Amiodarone	
	K_d	*b	K_d	b
Liver	3.7±0.1x10^{-10}M	1.0±0.1	7.2±2.1x10^{-6}M	0.9±0.1
Brain			13.7±1.6x10^{-6}M	1.0±0.1
GC cell	5.6±1.7x10^{-10}M		1.6±0.2x10^{-6}M	0.7±0.1
	†EC_{50}		EC_{50}	

GC Nuclear Binding

(intact cells)

$8.7\pm0.2\times10^{-10}$M	1.0±0.1	$3.3\pm0.2\times10^{-6}$M	0.9±0.4

*b is a slope factor. Determined with Allfit program. Values for b did not differ significantly from 1.0.

Effective Concentration (EC_{50}) is the apparent concentration of agonist T3 or antagonist amiodarone needed to decrease binding of ^{125}-I T3 (300pM) to 50% maximum values.

Table 1C shows binding affinities (K_d) of T_3 and amiodarone to solubilized nuclear receptors of rat liver, brain, pituitary tumor cells or in intact cultured cells. K_d values were determined by competition binding studies and analyzed by weighted, non-linear least squares regression with the aid of the Scatfit computer program. Affinities derived from liver receptor preparations represent the weighted means from six experiments. Other values represent the mean of at least two experiments.

While amiodarone competed with ^{125}I-T3 for binding to solubilized receptors from pituitary tumor cells of the GC cell line with K_d of 1.6 μM, the affinity for receptors from rat brain of K_d 13.7 37 μM was smaller, suggesting that amiodarone interacted differently with the brain receptors than with GC cell receptors. When both T3 and amiodarone were incubated with intact cells, both T3 and amiodarone competed with labeled T3 for binding to the thyroid hormone receptors, as shown by their EC_{50} values in Table 1.

In a variety of solubilized receptor preparations, amiodarone competed with T3 for binding to a single class of sites in a dose-dependent fashion at concentrations from 10^{-7} to 10^{-4}M as shown in Figure 5. For rat liver receptor, the 50% inhibition (EC_{50}) occurred at 3μM amiodarone and 80% inhibition was at 50 μM.

Figure 5 shows a competition by unlabelled L-T3 and amiodarone for binding of L-125 I-T3 to rat nuclear thyroid hormone receptors solubilized from various tissues. Pictured are competition curves for T3 in liver receptors (open circles); and for amiodarone in liver (closed circles), whole brain (closed boxes), and GC cell receptors (closed triangles). The affinity (K_d) for T3 in liver nuclear receptors was 3.7×10^{-10}M. In the individual experiments shown, affinities for amiodarone were 1.4 μM, 13.7 μM and 2.9 37 μM in solubilized receptors from rat liver, brain and pituitary tumor (GC) cells, respectively. [R]-160-280 pM. ^{125}I-T3 is 100-200 pM.

The competitive nature of amiodarone binding was investigated by using 10 μM amiodarone on the binding of increasing doses of ^{125}I-T3 to soluble nuclear receptors, computer modeling of these curves revealed an amiodarone Ki of 9.3 μM for a single class of sites, which was not statistically different from 7.2 μM obtained with a fixed amount of ^{125}I-T3 and with varying concentration of amiodarone. Increasing concentrations of T3 reversed amiodarone's ffect on T3 binding which is characteristic of

a competitive binding inhibitor. The data shown in Figure 6 are redrawn as a Lineweaver-Burk plot.

Figure 6 shows effects of amiodarone on saturation binding T3 to soluble nuclear receptors. Figure 6a shows saturation binding isotherms where 10 pM of soluble rat liver nuclear receptor (3 pmol/mg) was incubated with increasing amounts of ^{125}I-T3 in the absence (K_d) and presence (K_d') of inhibitor, and these values were used to determine a K_i for amiodarone. In these experiments more pure preparations of rat liver receptor were utilized than before. The following values were obtained from computer modeling: K_d=108 pM, K_d'=224pM, and K_i=9.3 μM. The lower line (closed triangles) indicates non-specific binding (average of both curves), which represented 2-4% of total binding. Binding of ^{125}I-T3 in the presence of 1 μM unlabeled T3 yields values for non-specific binding (NSB). Separation of bound from free T3 was achieved with Sephadex. Figure 6b shows Lineweaver-Burk transformation of the data. The abscissa (x) and ordinate (y) represent the inverse of free pM ^{125}I-T3 and pM ^{125}I-T3 specifically bound, respectively. The slopes (b) of the regressions in the absence and presence of amiodarone were 0.07±0.00 and 0.13±0.00, respectively (b±95% C.I.). The calculated K_i from these slopes using the less accurate graphical methods was 11.6 μM. The y-intercepts (a) were 6.6±0.2 and 6.8±0.7 (a±SEE), respectively, indicating that maximum binding did not differ in the absence or presence of amiodar ne. r^2=0.99 for each regression line.

The GC culture cells were further utilized to assess the effect of the amiodarone on T3 action. These cells respond to T3 by increasing their synthesis of rat growth hormone (rGH) due to increases in rGH mRNA resulting from transcriptional activation of the growth hormone gene. As shown in Figure 7a, amiodarone blocked the induction of rGH mRNA by 300 pM T3 as analyzed by RNA blot using Northern analysis. Figure 7b displays an amiodarone mRNA competition curve and as can be seen, the EC_{50} for blockade of rGH mRNA by amiodarone was 3.1 μM, very similar to the EC_{50} for binding to the thyroid hormone receptor in the presence of 300 pM T3, identical experimental conditions.

Figure 7 shows effects of amiodarone on accumulation of rGH mRNA and on binding by T3 to nuclear fractions of cultured GC cells.

Figure 7A is a autoradiogram of a representative Northern blot of rGH nRNA from GC cell cytoplasm. GC cells were plated at 2-4x10^6 cells/100 mm tissue culture dish (Falcon) and were maintained at after four days of deinduction, cells were cultured for an additional 2 days in the presence of stripped media containing 300 pM T3 plus varying doses of amiodarone as described above.

Figure 7B, open circles shows composite mRNA response curve constructed from 4 amiodarone experiments with cultured GC cells. After treatment with T3 and graded doses of amiodarone, cytoplasmic RNA was prepared and analyzed by Northern hybridization as described in above. After

hybridization, blots were autoradiographed and analyzed using Zenith scanning densitometer. Arbitrary density units were used to construct competition curves and to derive EC_{50} values and slope factors for amiodarone's effect on rGH mRNA accumulation. The "100%" point on the curve represents data from cells treated with 300 pm T3 without amiodarone. The circled point represents data from non-treated cells.

Closed circles show amiodarone inhibition of binding of ^{125}I-T3 to nuclei of intact GC cells.

These results of various pharmacological and biological tests show that amiodarone acts as a competitive antagonist to thyroid hormone action. These results also show that amiodarone binds to thyroid hormone receptors from a variety of tissues and inhibits T3 induced increases in growth hormone mRNA levels in GC cells in a similar dose-dependent manner, and are in agreement with the discovery that spatial atom arrangements, determined in Example 3, lower portion of the amiodarone are similar to that of T3, but spatial atom arrangement of amiodarone's upper portion is dissimilar to T3.

EXAMPLE 5

Evaluation of Compounds' Pharmacological Activity

This example illustrates the utility of the current invention for evaluation and/or prediction of compounds pharmacological activity.

Representative compounds from groups of R^1 and R^2 substituents were chosen to be evaluated first by the procedure described in Example 3. Their coordinates were

determined and input into VAX computer and the three dimensional models wer prepared. Then the structural three dimensional models were superimposed on the T3 model structure and both models were manipulated to prove or disprove the spatial fit. When the fit was there, a binding study to the T3 receptor was performed.

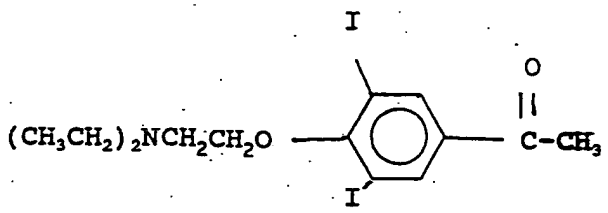
Only when both the spatial fit and receptor binding was found, the compound was recommended for further pharmacological testing to determine its possible pharmaceutical utility.

For the compounds listed below, receptor binding studies were performed in a manner entirely analogous to the examples previously given, with the exception of the source of the receptor. In Figures 8-13, human thyroid hormone receptor was produced by in-vitro translation of RNA coding for the receptor protein. The RNA was synthesized enzymatically using a human thyroid receptor DNA clone as a template. Such a system has been described previously as in Nature, Vol. 324, p 635-640 and p 641-646 (1986).

Figures 8-13 illustrate the thyroid receptor binding results.

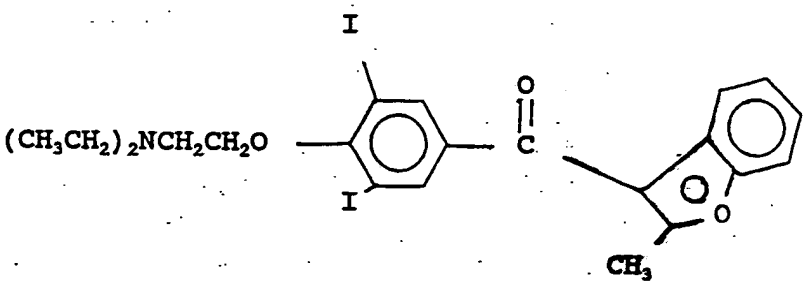
Figure 8 serves as a standard with ^{125}I-T3 and amiodarone, which was already shown to have spatial fit and receptor binding, see Figures 2A and 2B.

1. Figure 9 shows the following compound:



This compound structurally fitted when its model was superimposed over the structure (1) model. When however submitted to the receptor binding assay, there was zero binding. For Figures 8-13, ☐ show ^{125}I-T3, ◆ show experimental compound. The compound was not recommended for further pharmacological testing.

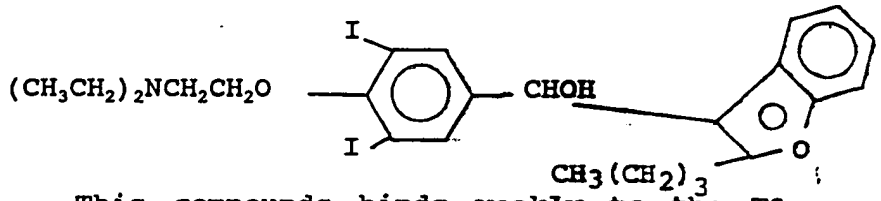
2. Figure 10 shows the following compound:



This compound spatially fits when superimposed model T3, but substitutes a methyl for the butyl group of amiodarone. This compound has spatial fit when superimposed over the T3 model volume (structure 1) and also shows good binding to T3 receptor.

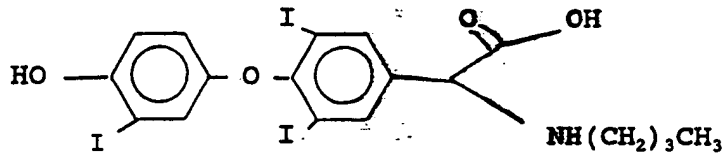
It was strongly recommended for further pharmacological testing.

3. Figure 11 shows the following compound:



This compounds binds weakly to the T3 receptor, but since it binds, it was recommended for further testing with the expectation that either agonist or antagonist function will be found.

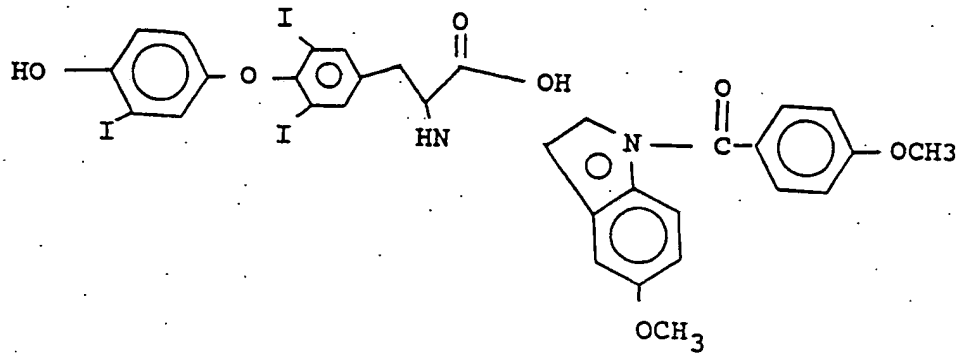
4. Figure 12 shows the following compound:



This compound fits within the spatial arrangement of the space model, structure (1) and binds to the T3 receptor.

It was recommended for further pharmacological testing.

5. Figure 13 shows the following compound:



This compound fits within the spatial arrangement of the space model, structure (1) and binds to the T3 receptors.

It was recommended for further testing.

Similarly to the compounds 1-5, all other compounds are tested first for their spatial fit and to the structure (1), and then for their binding property to the T3 receptor.

EXAMPLE 6

General Utilization of the Spatial Fits and Receptor Binding for Pharmaceutical Industry

This example illustrates the general utilization of the method of this invention for evaluation, prediction and design of the pharmacological activity of known or novel compounds of which structure can be, via its coordinates, entered into the computerized system of this invention and

compared to any standard compound of which the spatial model can be modelled into three dimensional spatial model and which is active as either agonist or antagonist of receptors which are isolated and for which the binding assay is available.

While only a few embodiments of the invention have been shown and described herein, it will become apparent to those skilled in the art that various other modifications and changes can be made a) in the present method to select compounds useful as pharmacologically active agents in a mammal, b) to the compounds themselves, c) their method of preparation, and d) their pharmaceutical use without departing from the spirit and scope of the present invention. All such modifications and changes are intended to be within the scope of the invention and appended claims.

WHAT IS CLAIMED IS:

1. A method for discovering and evaluating pharmacological activity of an experimental compound comprising:

(a) selecting a standard compound having known chemical structure and known pharmacological activity;

(b) preparing a three dimensional spatial atom arrangements model of the standard compound;

(c) preparing a three dimensional spatial atom arrangements model of the experimental compound;

(d) superimposing the model of the experimental compound over the model of the standard compound;

(e) determining three dimensional spatial similarities or dissimilarities of both compounds; and

(f) confirming spatial similarities with a pharmacological test.

2. The method of Claim 1 wherein the models of standard and experimental compounds are preparing by orienting the compounds into a conventional cartesian three dimensional x,y,z coordinate space axes planes.

3. The method of Claim 2 wherein the three dimensional model of experimental compound is superimposed over the three dimensional model of the standard compound and both structures are oriented along their vertical y axis until

their spatial similarities or dissimilarities are shown.

4. The method of Claim 3 wherein the similarities are due to the compound's atoms van der Waals characteristics.

5. The method of Claim 4 wherein the orienting of the compound structure, preparing three dimensional models, superimposing models and orienting models by rotation into conformational spaces is done by computer.

6. The mehod of Claim 5 wherein the pharmacological activity of the standard compound is a receptor agonist or antagonist.

7. The method of Claim 6 wherein the pharmacological activity of the standard compound is determined by receptor binding assay.

8. The method of Claim 7 wherein the pharmacological activity of the experimental compound is determined by the binding assay of Claim 7 to be the same as the pharmacological activity of the standard compound.

9. A method for designing a pharmacological activity of the experimental compound comprising:

(a) selecting a standard compound having known chemical structure and known pharmacological activity;

(b) preparing a three dimensional spatial atom arrangements model of the standard compound;

(c) preparing a three dimensional spatial model of the experimental compound, based on x,y,z coordinates of the standard compounds, with novel substituents wherein said spatial model fits within the three dimensional spaces occupied by the model of the standard compound;

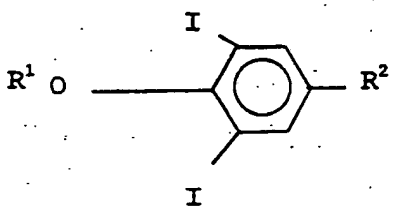
(d) determining the spatial fit by orientation of both standard and experimental compounds models along their vertical y axes;

(e) choosing the experimental compound having the conformational fit; and

(f) confirming the pharmacological activity of the experimental compound by a pharmacological test.

10. A method for selecting compounds having useful pharmacological properties in a mammal, which method comprises:

(a) selecting compounds of structure (1):



(1)

wherein R^1 and R^2 are each independently selected from aliphatic moieties, substituted aliphatic moieties, aromatic moieties or substituted aromatic moieties,

(b) orienting the structure (1) of step (a) into a conventional cartesian three dimensional x, y, z coordinate space axes in a manner such that the plane of the phenyl/iodine atoms is in the x, y plane and the carbon atom in the phenyl ring in the para-position to the attached oxygen atom is fixed on the 0,0,0-coordinate and the phenyl ring is bisected by the minus y axis, having standard distances between atoms based on the carbon-carbon single bond of 1.54 angstroms, wherein R^1 is spatially within space A which is defined as between plus 3.5 and minus 3.5 angstroms on the x-axis, between minus 5.1 and minus 16 angstroms on the y-axis, and between plus 3 and minus 8 angstroms on the z-axis, and

R^2 has van der Waals spatial characteristics comprising spaces B^1 and B^2,

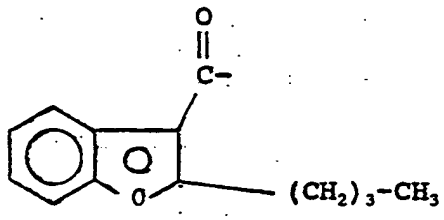
wherein B^1 is that area falling outside B^2, and having coordinates:

B^{1a} is the space within minus 4.3 to plus 5.2 angstroms on the x-axis, plus 4.5 to plus 8.5 angstroms on the y-axis, and minus 3.5 to plus 3.5 angstroms on the z-axis; and B^{1b} is the space within 0 and minus 5.0 angstroms on the x-axis, minus 5.1 and plus 4.5 angstroms on the y-axis and plus 4 and minus 3 angstroms on the z-axis, B^{1c} is the space within 0 and 6 angstroms on the x-axis, minus 5.1 and plus 4.5 angstrom on the y-axis and 0 and minus 3 angstroms on the z-axis, and

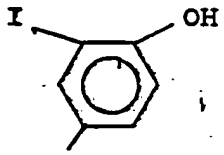
B^2 is between zero and plus 10 angstroms on the x-axis, between minus 5.1 and plus 4.5 angstroms on the y-axis and

between zero and plus 10 angstroms on the z-axis,

with the proviso that R^1 and R^2 are not both unsubstituted methyl or ethyl or a combination thereof, and when R^2 is $-CH_2CH_2N(CH_2CH_3)_2$ or $-CH_2CH_2NHCH_2CH_3$, R^2 is not



or when R^1 is



R^2 is not $HOC(=O)CH(NH_2)CH_2-$.

(c) performing receptor binding study.

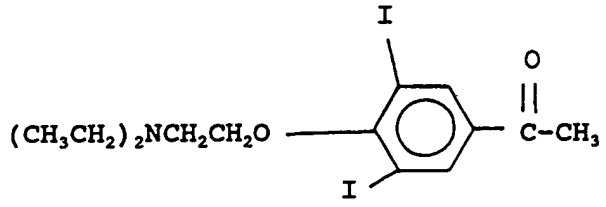
11. The method of Claim 10 wherein in the orienting of the 3-dimension structure to select compounds uses commercial computer programs identified as "MM2", "SYBIL", "BIOGRAF", "MIDAS", or combinations thereof.

12. The method of Claim 10 wherein the pharmaceutical properties are useful to treat a condition selected from hyperthyroidism, angina pectoris, antiadrenergism, arrythmia or cardiac ischemia, anxiety or obesity.

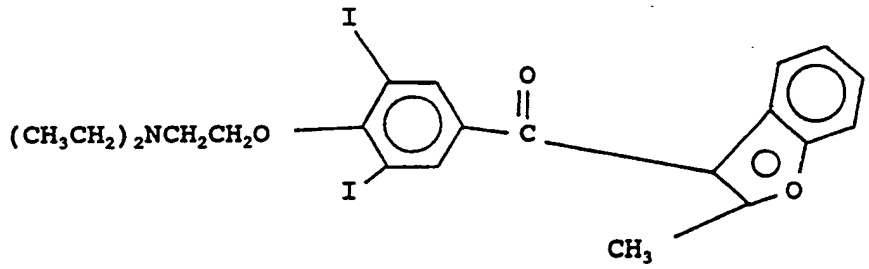
13. The method of Claim 10 wherein the useful pharmaceutical property is used in the treatment of

14. The method of Claim 10 wherein R^1 and R^2 contain at least one polar atom selected from O, S, N or combinations thereof.

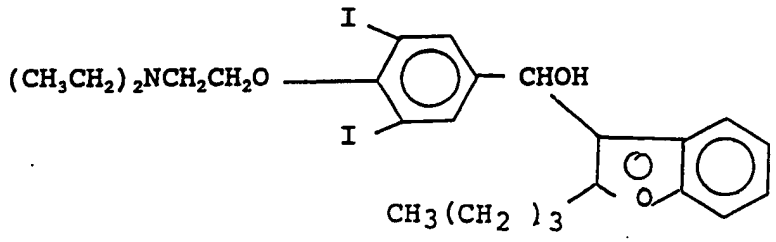
15. The compound of structure:



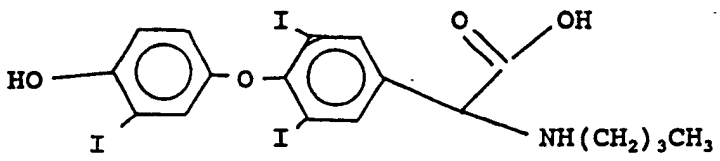
16. The compound of structure:



17. The compound of structure:



18. The compound of the structure:



19. The compound of the structure:

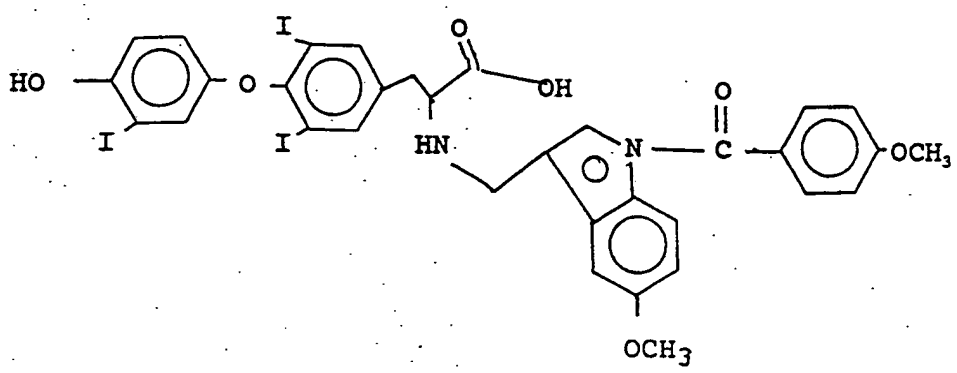
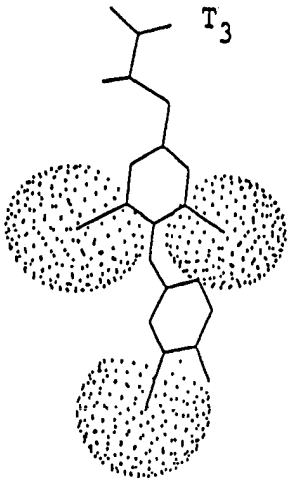


FIGURE 1A



AMIODARONE

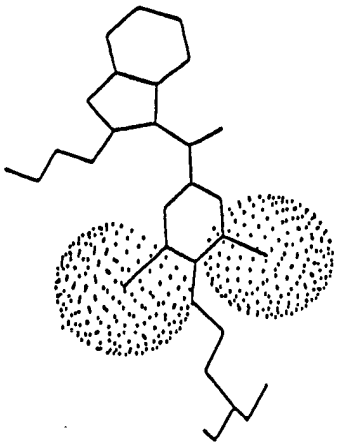
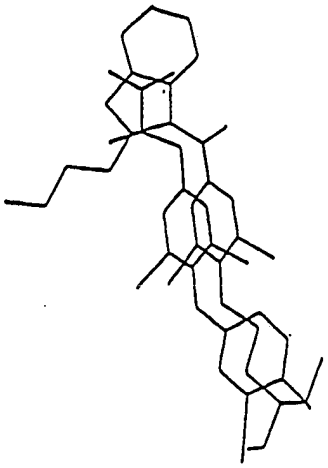


FIGURE 1B



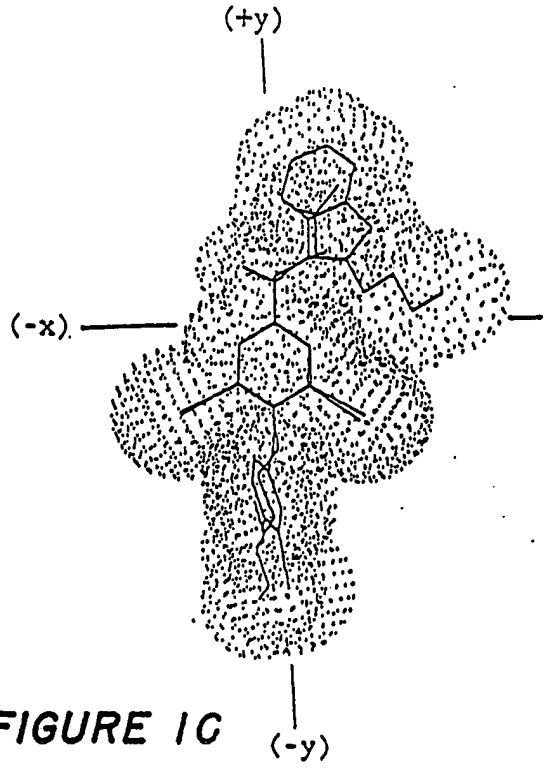


FIGURE 1C

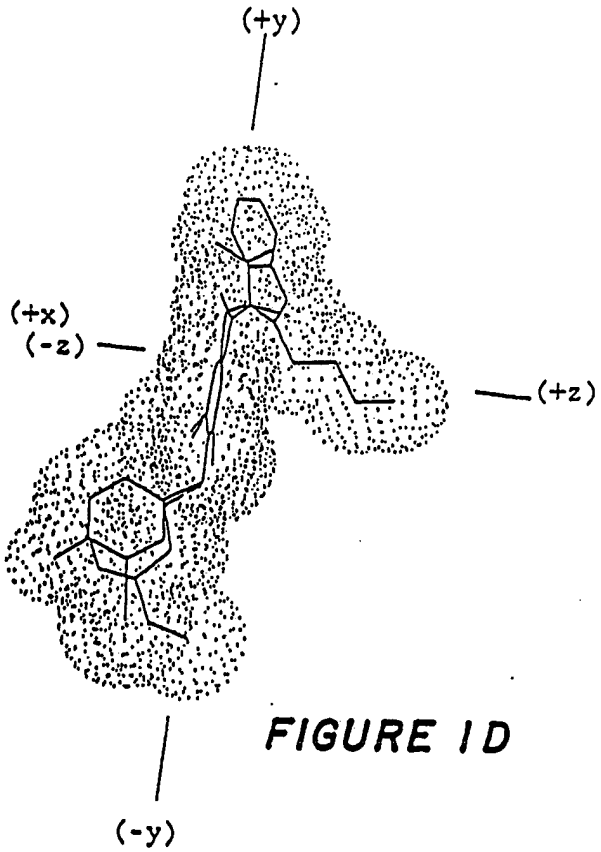


FIGURE 1D

FIGURE 1

FIGURE 2B

Triiodothyronine
(MW:651)

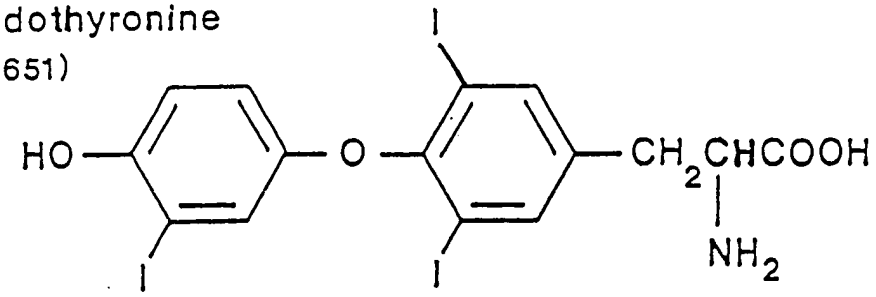


FIGURE 2A

Amiodarone
(MW:645)

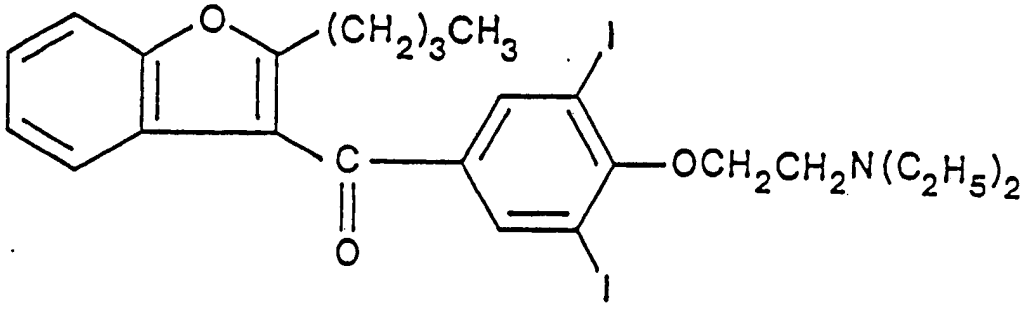


FIGURE 2

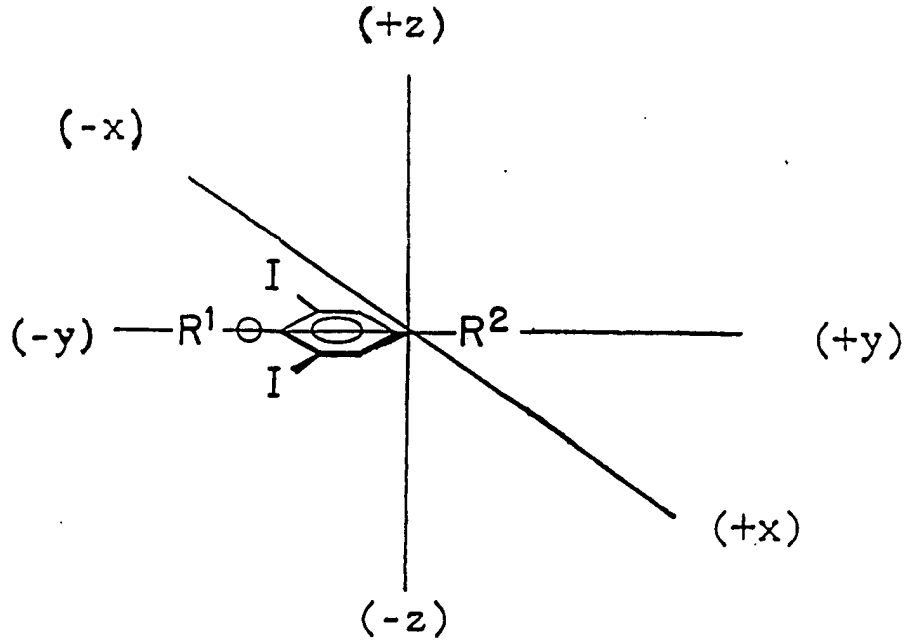


FIGURE 3

FIGURE 4A

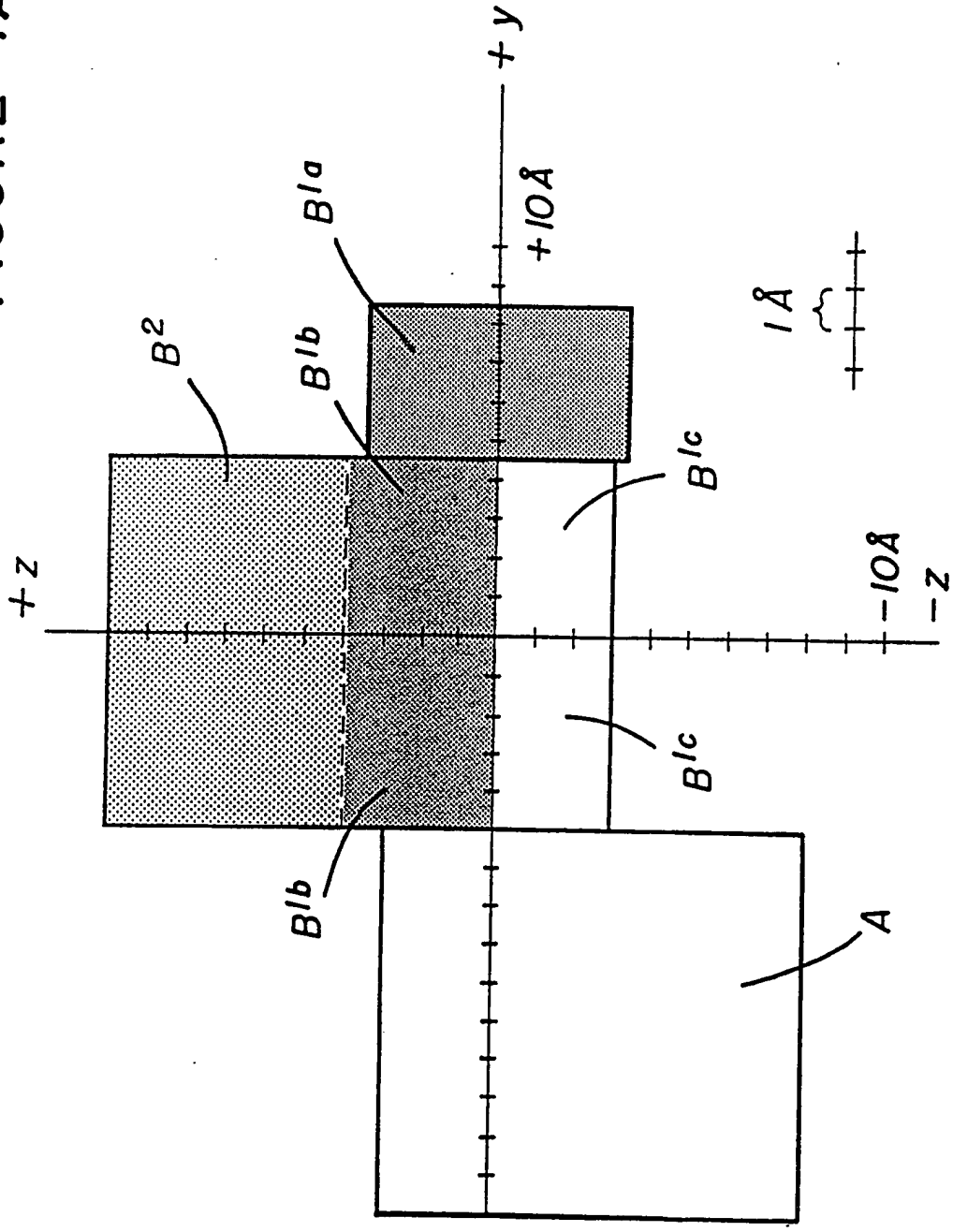
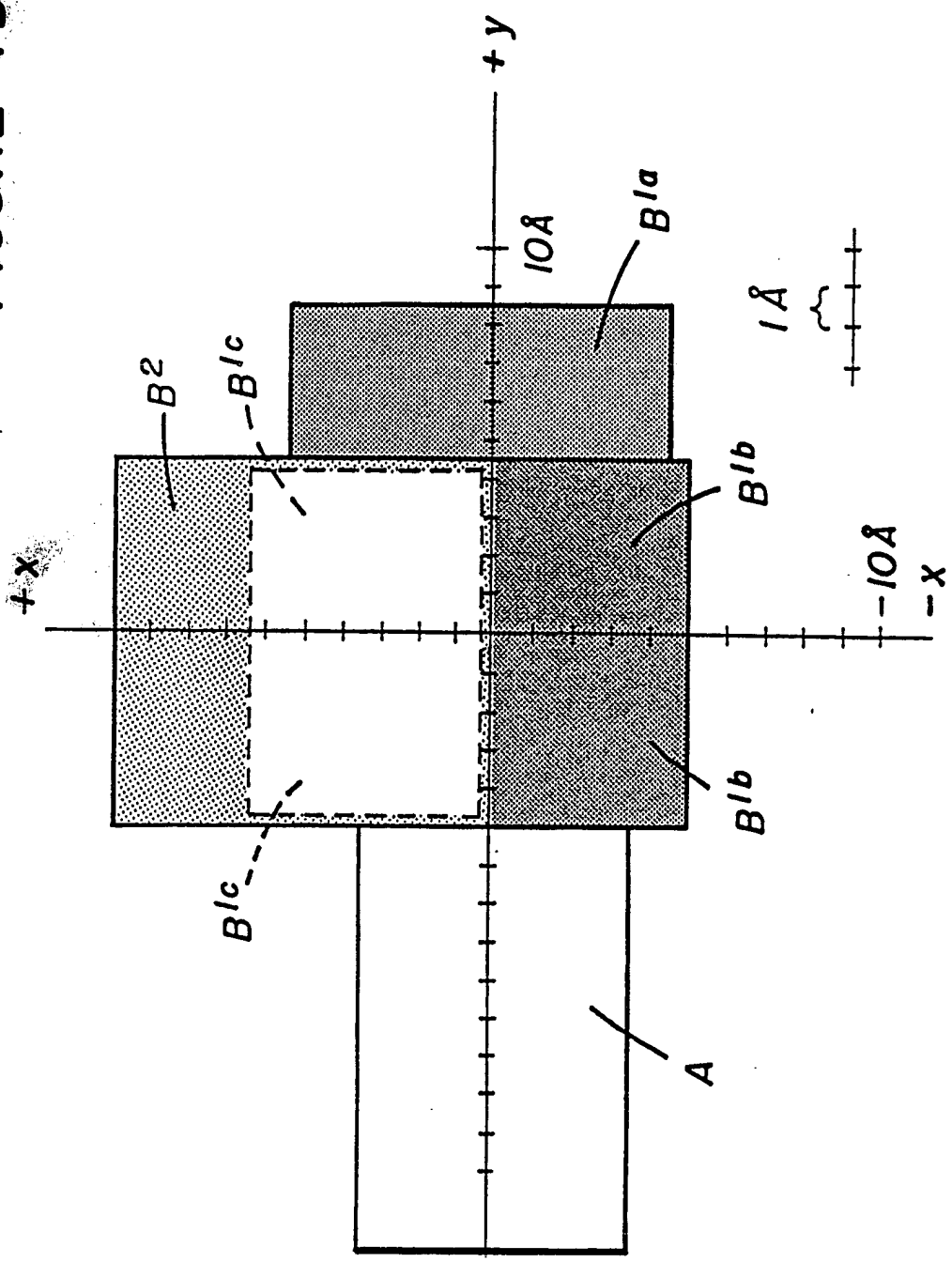


FIGURE 4B



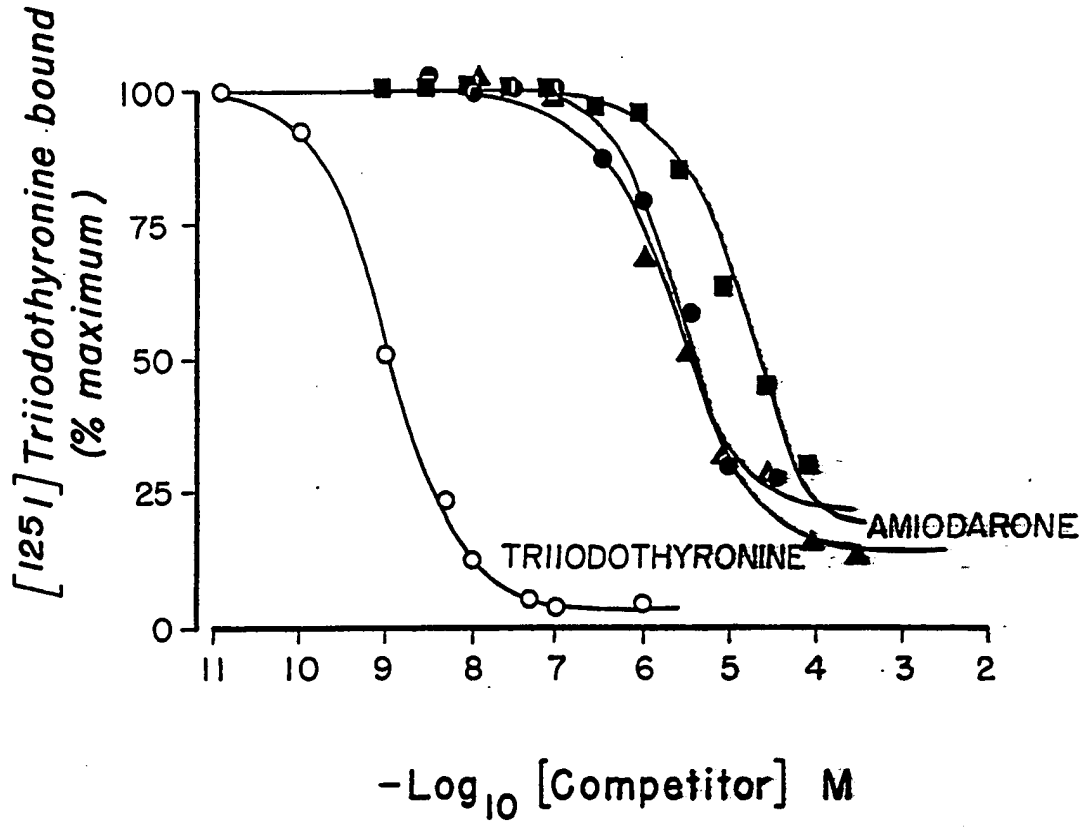

[125I] Triiodothyronine bound
(% maximum)
100
75
50
25
0
11
10
9
8
7
6
5
4
3
2
AMIODARONE
TRIIODOTHYRONINE
-Log10 [Competitor] M

FIGURE 5

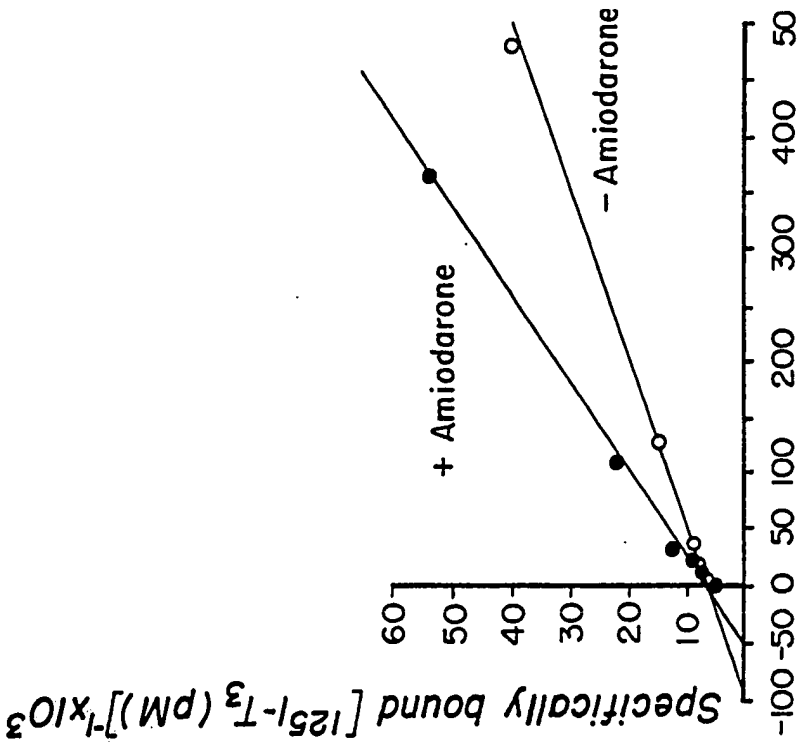


FIGURE 6 B

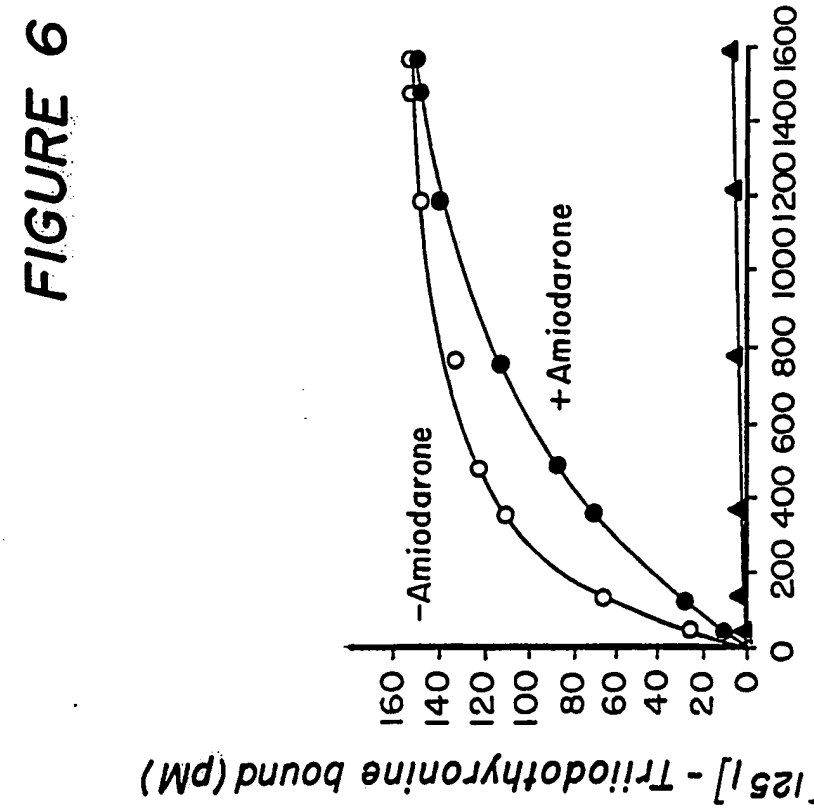


FIGURE 6A

FIGURE 6

FIGURE 7

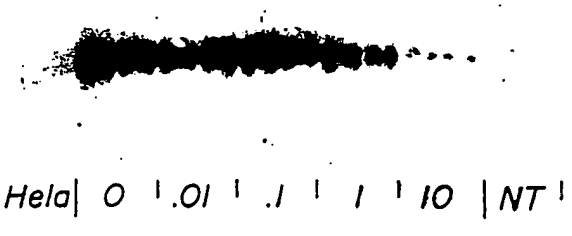


FIGURE 7A

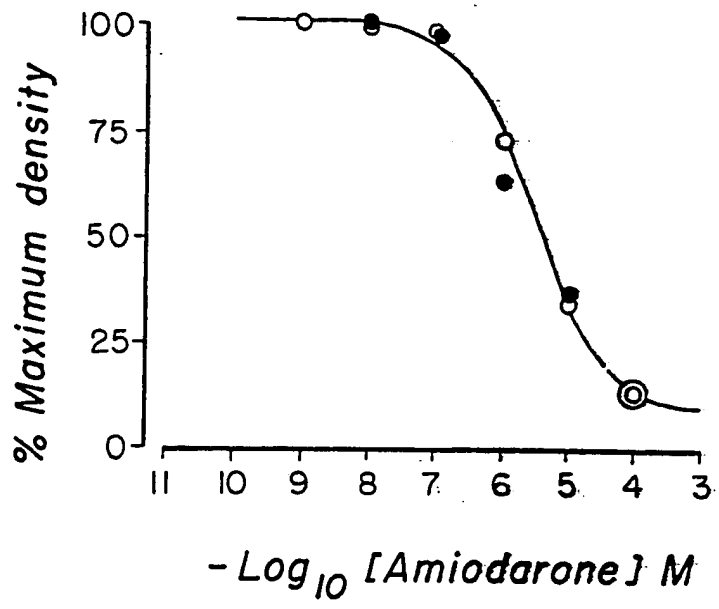


FIGURE 7B

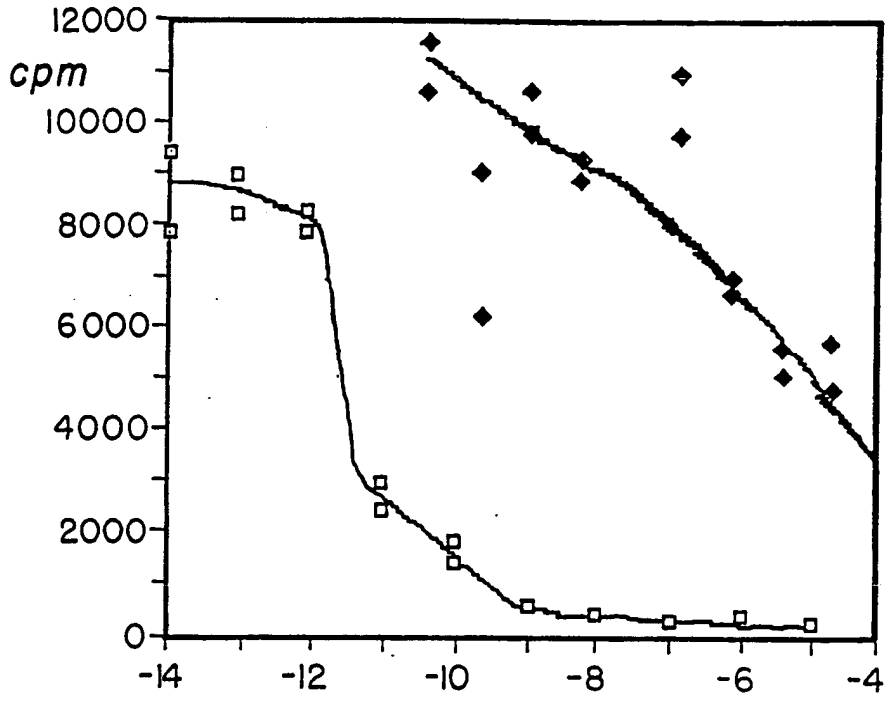


FIGURE 8

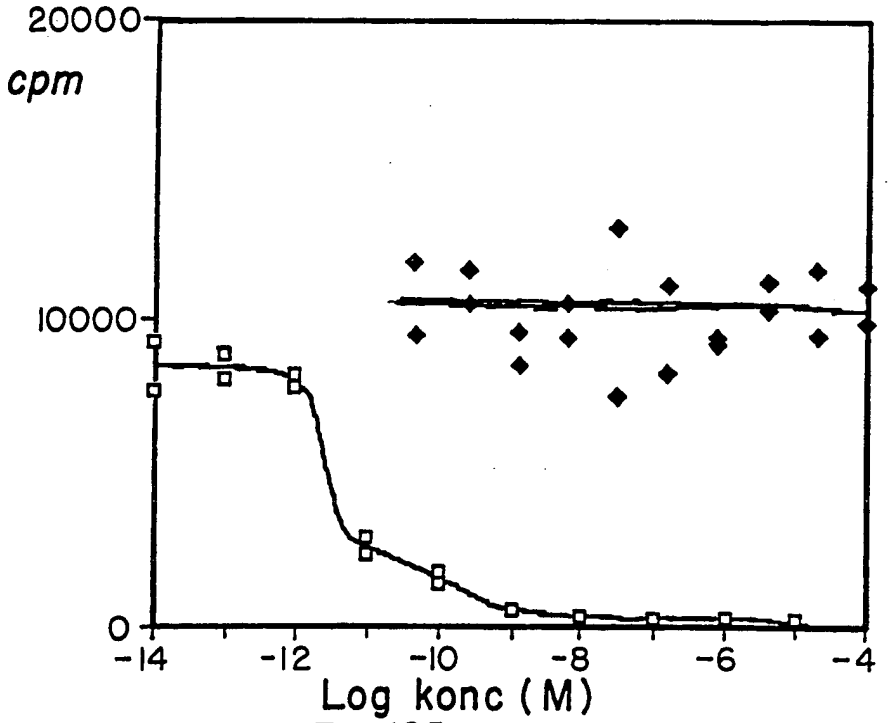


FIGURE 9

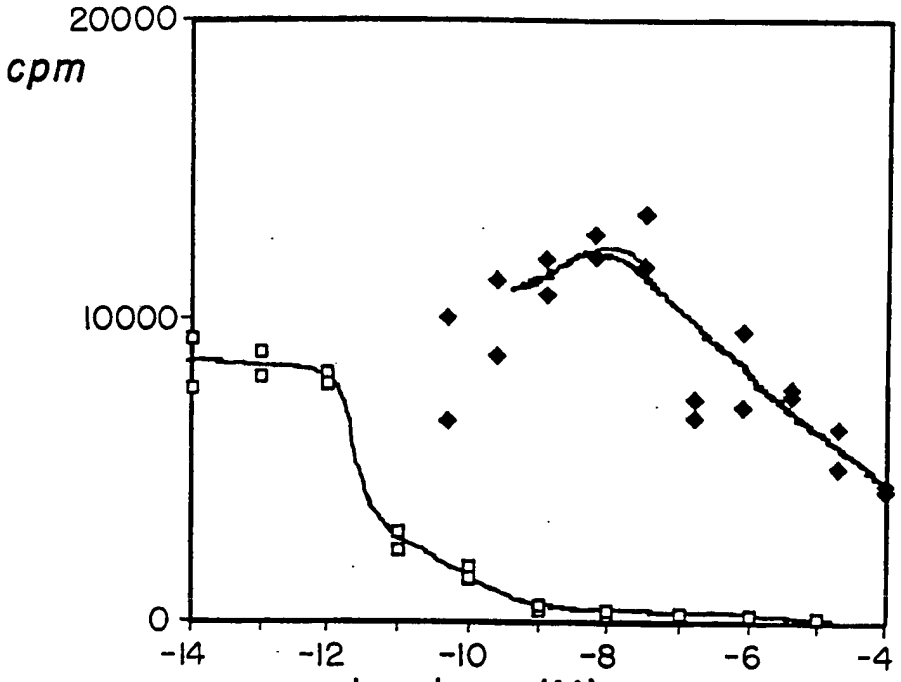


FIGURE 10

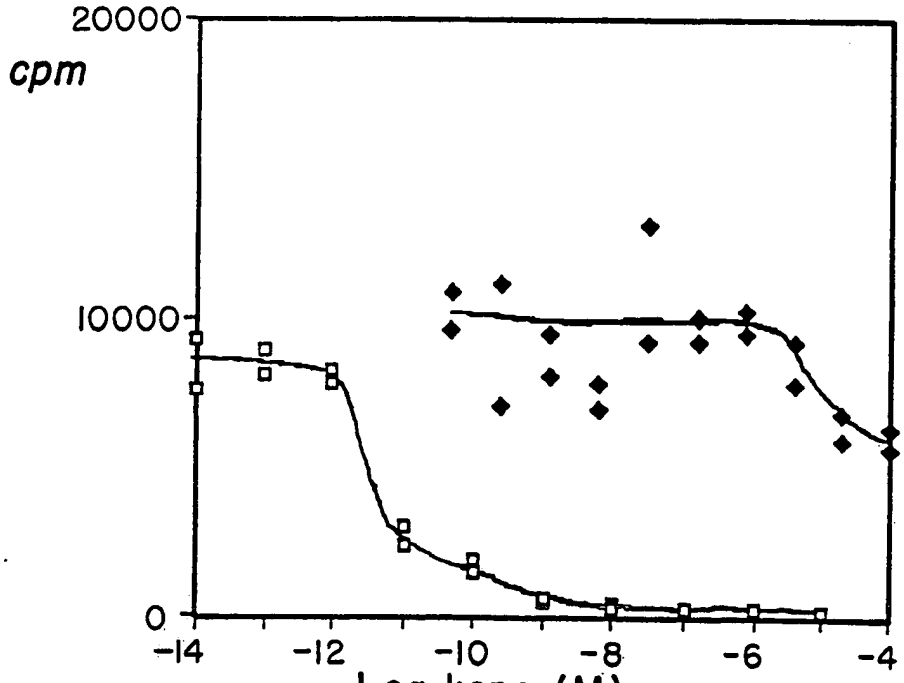


FIGURE 11

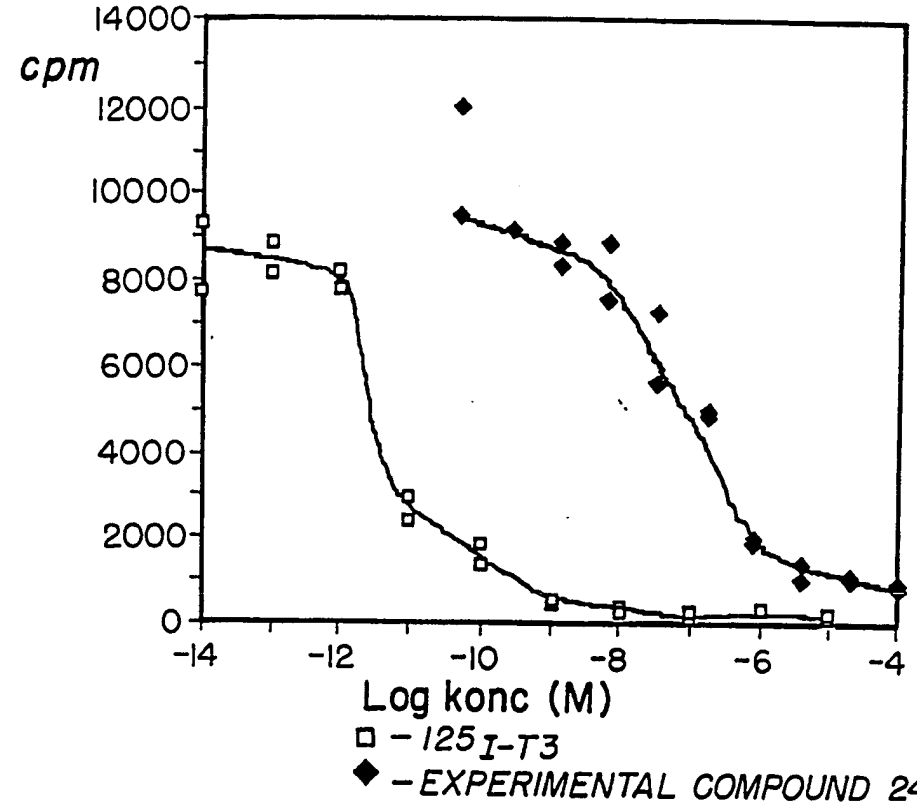


FIGURE 12

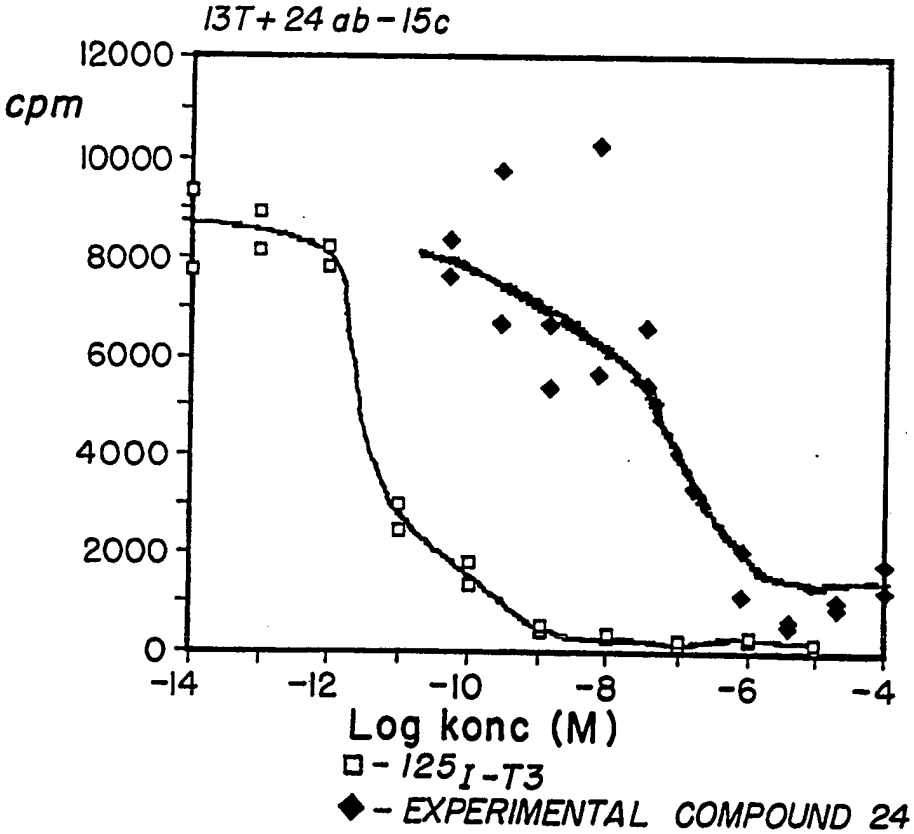


FIGURE 13

INTERNATIONAL SEARCH REPORT

International Application PCT/US90/00108

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 31/19; A61K 31/00

U.S.Cl.: 514/568; 514/570; 514/821

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	514/568; 514/570; 514/821

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	US, A, 4,363,815 (YU ET AL) 14 December 1982, see the entire document.	1-19
Y	GB, A, 980,276 (EGEMA) 13 January 1965, see the entire document.	1-19
Y	GB, A, 1,587,638 (SCHIBLI) 8 April 1981, see the entire document.	1-19
Y	Chemical Abstracts, Volume 68, No. 1, issued 9 January 1968 (Columbus, Ohio, USA, A. Baudine, "Benzofuran derivatives. XXVI. General Pharmacological Effects of Amidarone", see page 161, column 2, the abstract no. 1848y, Arch. Int. Pharmacodyn. Ther 1967, 169(2), 469-81 (Fr)).	1-19
Y	FR, M, 8005 (PACHECO ET AL) 27 July 1970 (see the abstract).	1-19
Y	FR, M, 8256 (PACHECO ET AL) 30 November 1970 (see the abstract).	1-19

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 FEBRUARY 1990	18APR 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	KEVIN E. WEDDINGTON

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Chemical Abstracts, Volume 89, No. 11, issued 21 September 1978 (Columbus, Ohio, USA, D.P. Zipes, "New antiarrhythmic agents", see page 1, column 2, the abstract no. 84419s, American Journal Cardiology. 1978, 41(b), 1005-24 (Eng).	1-19

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.